REGULATION OF BIOFILM FORMATION

Statement as to Federally Sponsored Research

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Field of the Invention

The field of the invention is bacterial genetics.

Background of the Invention

Populations of surface-attached microorganisms, comprised either of single or multiple species, are commonly referred to as biofilms. In most natural, clinical, and industrial settings, bacteria are found predominantly in biofilms, not as planktonic cells such as those typically studied in the laboratory. Biofilm bacteria display a different gene expression pattern, different cellular physiology, and higher resistance to antibiotics, relative to their planktonic counterparts.

Numerous reports have documented the ability of diverse bacterial species to form biofilms on a variety of abiotic surfaces of great importance in medicine and industry. For example, *Pseudomonas aeruginosa*, an organism that causes nosocomial infections, forms biofilms on surfaces as diverse as cystic fibrosis lung tissue, contact lenses, and catheter lines. In general, biofilms can become hundreds of microns in depth, thereby clogging tubular structures such as catheters and industrial pipes.

Biofilm development initiates when bacteria make the transition from a planktonic existence to a lifestyle in which the microorganisms are firmly attached to biotic or abiotic surfaces. This transition is thought to be regulated in part by the nutritional status of the environment. After their initial attachment to the substratum, the cells are believed to undergo a program of physiological changes that result in a highly structured, sessile microbial community. After growth and development of the biofilm, the developmental cycle is completed when planktonic cells are shed from the biofilm into the medium, perhaps in response to a lack of sufficient nutrients (Costerton, J.W., et al.,1995, In *Annu. Rev. Microbiol.* Ornston, L.N., *et al.* (eds.). Palo Alto, CA: Annual Reviews, Inc., pp. 711-745; Wimpenny, J.W.T. and Colasanti, R.,1997, *FEMS Microbiol. Ecology* 22: 1-16).

Previous studies exploring biofilm formation have generally focused on identification of the organisms that comprise biofilms, their physical and chemical properties, and biofilm architecture (Costerton, J.W., et al.,1995, *supra*). In contrast, little is known about the cellular factors and molecular mechanisms required for the transition from a planktonic to a sessile mode of life and the subsequent development of a biofilm.

Understanding the molecular factors that contribute to biofilm initiation and maintenance would allow us to better control biofilm formation, and would thereby have a significant impact upon medicine, industry, and the environment.

Summary of the Invention

Using *Pseudomonas fluorescens*, *Escherichia coli*, and *Pseudomonas aeruginosa* as model organisms, we have investigated the molecular mechanisms required for biofilm formation. We have identified nutritional

conditions that modulate biofilm formation in wild-type bacteria and in mutants defective in biofilm formation, and have identified a class of genes involved in biofilm formation, designated *s*urface *a*ttachment *d*efective (*sad*). The *sad* genes, *sad* gene products, and *sad* transcriptional control regions may all be used for the control of biofilm formation in commercially important fields such as manufacturing, agriculture, and healthcare. Furthermore, these reagents may be used in methods for the detection of industrially and pharmaceutically useful compounds for the modulation of biofilm formation.

In a first aspect, the invention features a purified nucleic acid. The purified nucleic acid includes a region that hybridizes under high stringency conditions to a probe containing at least 75 consecutive nucleotides that are complementary to a portion of an n-*sad* gene, wherein the region contains at least 75 consecutive nucleotides. In preferred embodiments of this aspect of the invention the n-*sad* gene is a *P. fluorescens sad* gene including a sequence chosen from SEQ ID NOs: 1-24, or the nucleic acid is contained within an expression vector.

In another preferred embodiment of the first aspect of the invention, the nucleic acid encodes a polypeptide that has a biological activity necessary for biofilm formation under at least one condition known to allow biofilm formation by a bacterium that expresses said polypeptide.

In a second aspect, the invention features a probe comprising at least 18 nucleotides that are complementary to an n-*sad* gene from *P. fluorescens* including a sequence chosen from SEQ ID NOs: 1-24. In preferred embodiments of this aspect of the invention, the probe includes at least 25, 40, 60, 80, 120, 150, 175, or 200 nucleotides that are complementary to the n-*sad* gene.

In a third aspect, the intention features a substantially pure n-*sad* polypeptide. In a preferred embodiment of the second aspect of the invention, the polypeptide has a biological activity necessary for biofilm formation under at least one condition known to allow biofilm formation by a bacterium that expresses the polypeptide.

In a fourth aspect, the invention features a substantially pure antibody that specifically binds an n-*sad* polypeptide.

In preferred embodiments of the third and fourth aspects of the invention, the polypeptide includes a polypeptide encoded by a *P. fluorescens* n-*sad* gene that includes a sequence chosen from SEQ ID NOs: 1-24.

In a fifth aspect, the invention features a method of screening for a compound that modulates biofilm formation including a) contacting a sample containing a *sad* gene, *sad*/reporter gene fusion, or *sad* polypeptide with a test compound, and b) measuring the level of *sad* biological activity in the sample. An increase in *sad* biological activity in the sample, relative to *sad* biological activity in a sample not contacted with the test compound, indicates a compound that increases biofilm formation. A decrease in *sad* biological activity in the sample, relative to *sad* biological activity in a sample not contacted with the test compound, indicates a compound that decreases biofilm formation. In preferred embodiments, the sample comprises bacterial cell extract; the *sad* gene, the *sad*/reporter gene, or the *sad* polypeptide is within a bacterial cell; the *sad* gene, the *sad*/reporter gene, or the *sad* polypeptide are from *P. fluorescens*, and the *sad* gene and the *sad*/reporter gene include a sequence chosen from SEQ ID NOs: 1-24, or the *sad* polypeptide is encoded by a gene comprising a sequence chosen from SEQ ID NOs: 1-24.

In a sixth aspect, the invention features a method of screening for a compound that modulates biofilm formation including a) contacting a sample with a test compound, wherein the sample contains a *clpP* gene, a *clpP*/reporter gene, or a ClpP polypeptide, and b) measuring the level of ClpP activity in the sample. An increase in ClpP activity in the sample, relative to ClpP activity in a sample not contacted with the test compound, indicates a compound that increases biofilm formation. A decrease in ClpP activity in the sample, relative to ClpP activity in a sample not contacted with the test compound, indicates a compound that decreases biofilm formation. In preferred embodiments, the sample comprises bacterial cell extract; the *clpP* gene, the *clpP*/reporter gene, or the ClpP polypeptide is within a bacterial cell; the *clpP* gene, the *clpP*/reporter gene, or the ClpP polypeptide is from *P. fluorescens*; the ClpP activity is measured by measuring biofilm formation; or the *clpP* gene, *clpP*/reporter gene, or ClpP polypeptide is a non-*E. coli clpP* gene, a non-*E. coli clpP*/reporter gene, or a non-*E. coli* ClpP polypeptide.

In another preferred embodiment of the sixth aspect of the invention, the *clpP* gene, *clpP*/reporter gene, or ClpP polypeptide is within a bacterial cell and the bacterial cell is cultured under standard biofilm assay conditions after the contacting.

In a seventh aspect, the invention features a method for preventing a bacterial cell from participating in formation of a biofilm. The method may include any one of the following: inhibiting the synthesis or function of a *sad* polypeptide; inhibiting protein synthesis in the bacterial cell; contacting bacterial cell with a protease, where the contacting is sufficient to prevent the bacterial cell from participating in formation of a biofilm; limiting the concentration of Fe^{2+}/Fe^{3+} in the environment of the bacterial cell, where the Fe^{2+}/Fe^{3+} concentration of the environment is limited to 0.3 μM or less;

providing a high osmolarity environment to the bacterial cell, where the osmolarity of the environment is equivalent to or greater than the osmolarity of a solution containing 0.2 M NaCl or 15% sucrose; and adding mannose to the environment of the bacterial cell, such that the mannose concentration in the environment after the addition of the mannose is at least 15 mM; and adding α-methyl-D-mannoside to the environment of the bacterial cell, such that the α-methyl-D-mannoside concentration in the environment after the addition of the α-methyl-D-mannoside is at least 15 mM.

In preferred embodiments the *sad* polypeptide is encoded by a *P. fluorescens sad* gene; the mannose concentration or the α-methyl-D-mannoside concentration is at least 15 mM, 25 mM, 50 mM, or most preferably 100 mM; or the surface is an abiotic surface.

In further embodiments of aspects 5, 6, and 7, the bacterial cell is selected from the group including: *Pseudomonas fluorescens, Pseudomonas aeruginosa, Escherichia coli, Vibrio paramaemolyticus, Salmonella typhimurium, Streptococcus mutans, Enterococcus* species, *Serratia marcescens, Staphylococcus aureus, Staphylococcus epidermidis,* and other coagulase-negative *Staphyloccus* species, such as *S. hominis, S. haemolyticus, S. warneri, S. cohnii, S. saprophyticus, S. capitis,* and *S. lugdunensis.*

In an eighth aspect, the invention features a method for inhibiting participation of a bacterium in formation of a biofilm on a surface. The method includes inhibiting the synthesis or function of a flagellum on the bacterium. In preferred embodiments the surface is abiotic; or the synthesis or function of the flagellum is inhibited by inhibiting the synthesis or function of: FliC (Genbank Accession No. L07387 (gb-L07387); SEQ ID NO: 34); FlhD (gb-AE000283, U00096; SEQ ID NO: 35); MotA (gb-J01652; SEQ ID NO: 36); MotB (gb-M12914; SEQ ID NO: 37); FliP (gb-L22182, L21994; SEQ ID NO: 38); FlaE

(gb-D90834, AB 001340; SEQ ID NO: 39); or FlgK (gb-AE000209, U00096; SEQ ID NO: 40); or homologues thereof. In another preferred embodiment of the eighth aspect of the invention, the inhibiting is under conditions that otherwise result in biofilm formation.

In a ninth aspect, the invention features a method for inhibiting participation of a bacterium in formation of a biofilm on an abiotic surface. The method includes inhibiting the synthesis or function of a pilus on the bacterium. In preferred embodiments the function of the pilus is inhibited by contacting the pilus with mannose or α-methyl-D-mannoside; the synthesis or function of the pilus is inhibited by inhibiting the synthesis or function of: PilB (Genbank Accession No. M32066 (gb-M32066); SEQ ID NO: 41); PilC (gb-M32066; SEQ ID NO: 42); PilD (gb-M32066; SEQ ID NO: 43); PilV (gb-L76605; SEQ ID NO: 44); PilW gb-L76605(; SEQ ID NO: 45); PilX (gb-L76605; SEQ ID NO: 46); PilY1 (gb-L76605; SEQ ID NO: 47); PilY2 (gb-L76605; SEQ ID NO: 48); or PilE (gb-L76605; SEQ ID NO: 49); or homologues thereof. In preferred embodiments, the bacterium is chosen from the group including: *Pseudomonas fluorescens, P. aeruginosa, Escherichia coli, Vibrio paramaemolyticus, Salmonella typhimurium, Streptococcus mutans, Enterococcus* species, *Serratia marcescens, Staphylococcus aureus, Staphylococcus epidermidis,* and other coagulase-negative *Staphyloccus* species, such as *S. hominis, S. haemolyticus, S. warneri, S. cohnii, S. saprophyticus, S. capitis,* and *S. lugdunensis.*

In a tenth aspect, the invention features a method of screening for a compound that inhibits bacterial pathogenicity. The method includes a) exposing a bacterial culture to a test compound, such that at least one bacterial cell in the bacterial culture is contacted by the test compound, and b) testing the bacterial culture for biofilm formation on an abiotic surface. A decrease in

biofilm formation, relative to biofilm formation by a bacterial culture that has not been exposed to the test compound, indicates a compound that inhibits biofilm formation, and an increase in biofilm formation, relative to biofilm formation by a bacterial culture that has not been exposed to the test compound, indicates a compound that stimulates biofilm formation. In preferred embodiments the bacterial culture is a liquid bacterial culture; at least 5%, 10%, 25%, 50%, 75%, or most preferably 100% of the bacterial cells contacted by the bacterial growth medium are contacted by the test compound; and the bacterial cell is chosen from the group including: *P. aeruginosa, Escherichia coli, Vibrio paramaemolyticus, Salmonella typhimurium, Streptococcus mutans, Enterococcus* species, *Serratia marcescens, Staphylococcus aureus, Staphylococcus epidermidis,* and other coagulase-negative *Staphyloccus* species, such as *S. hominis, S. haemolyticus, S. warneri, S. cohnii, S. saprophyticus, S. capitis,* and *S. lugdunensis.*

In an eleventh aspect, the invention features a method of stimulating formation of a biofilm by a population of bacteria. The method includes at least one of: adding iron to the growth environment of the bacteria, such that the final concentration of iron in the growth environment is at least 3 μM; adding glutamate to the growth environment of the bacteria, such that the final concentration of glutamate in the growth environment is at least 0.4%; adding citrate to the growth environment of the bacteria, such that the final concentration of citrate in the growth environment is at least 0.4%; and stimulating expression of a *sad* gene or activity of a *sad* polypeptide. In a preferred embodiment, the bacterium is *Pseudomonas fluorescens.*

By "biofilm" is meant a sessile population of microorganisms, comprised of a single species or multiple species, that are enclosed by an extracellular matrix and adhere to each other and to a biotic or abiotic surface.

By "standard biofilm assay" is meant experimental conditions that provide the equivalent to growth, on an abiotic surface, of approximately 10^6 colony forming units (CFU)/ml for 10 hours or 10^8 CFU/ml for approximately 30 minutes, at 30-37° C, preferably at 25° C, 30° C, or 37° C, in minimal M63 medium supplemented with 0.2% glucose and 0.5% casamino acids (CAA) or (particularly for *E. coli*) in rich medium such as Luria broth or Luria-Bertani broth.

By "environment" is meant the habitat or living conditions of a population of bacteria.

By "*sad* gene" or " surface attachment defective gene" is meant a DNA molecule that hybridizes at high stringency to one of the *sad* gene identifier sequences shown in Fig. 9, and encodes a polypeptide involved in biofilm formation on an abiotic surface under at least some environmental conditions. Examples of *sad* genes include the *P. fluorescens* genes *sad-10, sad-11, sad-13, sad-14, sad-16, sad-18, sad-19, sad-20, sad-21, sad-22, sad-51, sad-52, sad-53, sad-57, sad-58, sad-62, sad-79, sad-80, sad-81, sad-83, sad-87, sad-89, sad-98, sad-100, sad-101,* and *sad-102.*

By "*sad* polypeptide" is meant the protein product encoded by a *sad* gene.

By "n-*sad* gene" or "n-*sad* polypeptide" is meant a novel *sad* gene or gene product, including the *P. fluorescens* genes *sad-10, sad-11, sad-16, sad-18, sad-19, sad-20, sad-21, sad-22, sad-51, sad-52, sad-53, sad-57, sad-58, sad-62, sad-79, sad-80, sad-81, sad-83, sad-87, sad-89, sad-98, sad-100, sad-101,* and *sad-102,* and products of these genes.

By "*sad* gene identifier sequence" is meant a nucleotide sequence that constitutes a portion of a *sad* gene. A *sad* gene identifier sequence is at least 40 nucleotides, preferably at least 75 nucleotides, more preferably at least

125 nucleotides, and most preferably at least 175 nucleotides. *Sad* gene identifier sequences include SEQ ID NOs: 1-24, shown in Fig. 9.

By "*sad* mutant" is meant a bacterium that has a mutation in a *sad* gene and is defective for biofilm formation. A *sad* mutant may be defective for biofilm formation on only a subset of surfaces, or on all surfaces. For example, the *sad-10* mutant described below has a biofilm formation defect on hydrophobic surfaces such as PVC, polycarbon, and polypropylene, but forms biofilms indistinguishable from wild-type biofilms on a hydrophilic surface such as borosilicate glass.

By "reporter gene" is meant any gene that encodes a product whose expression is detectable and/or quantitatable by immunological, chemical, biochemical or biological assays. A reporter gene product may, for example, have one of the following attributes, without restriction: fluorescence (e.g., green fluorescent protein), enzymatic activity (e.g., lacZ/β-galactosidase, luciferase, chloramphenicol acetyltransferase), toxicity (e.g., ricin A), or an ability to be specifically bound by a second molecule (e.g., biotin or a detectably labelled antibody). It is understood that any engineered variants of reporter genes, which are readily available to one skilled in the art, are also included, without restriction, in the forgoing definition.

By "*sad*/reporter gene" or "*clpP*/reporter gene" is meant a DNA construct comprising transcriptional control sequences from, respectively, a *sad* gene or a *clpP* gene, operably linked to a reporter gene such that reporter gene expression is regulated in a manner analogous to that of an endogenous *sad* or *clpP* gene; therefore, modulation of expression of a *sad*/reporter or *clpP*/reporter gene construct, e.g., by a compound or environmental stimulus reflects modulation of expression of the endogenous *sad* or *clpP* gene. A

sad/reporter or *clpP*/reporter gene may exist within a cell as an episomal DNA molecule, or may be integrated into the cellular genomic DNA.

By "*sad*/reporter mRNA," "*sad*/reporter polypeptide," "*clpP*/reporter mRNA," and "*clpP*/reporter polypeptide," is meant, respectively, the mRNA or polypeptide encoded by a *sad*/reporter gene or a *clpP*/reporter gene.

By "changes in *sad* biological activity" is meant changes in: transcription of a *sad* gene or *sad*/reporter gene; post-transcriptional degradation or translation of a *sad* mRNA or *sad*/reporter mRNA; post-translational degradation, enzymatic function, or structural function of a *sad* polypeptide or *sad*/reporter polypeptide. In all cases, a change in *sad* biological activity in a sample, for example, a sample exposed to an environmental stimulus such as a change in nutrient status or the addition of a chemical, is measured by an increase or decrease, in the activity being measured, of at least 30%, more preferably at least 40%, still more preferably at least 55%, and most preferably by at least 70%, relative to a sample not exposed to the environmental stimulus.

By "ClpP polypeptide" is meant any protease that bears at least 70% sequence identity, more preferably at least 80%, and most preferably at least 89% sequence identity, over an amino acid stretch at least 50 amino acids in length, to the *P. fluorescens* ClpP polypeptide. One example of a ClpP polypeptide is the *E. coli* ClpP.

By "*clpP* gene" is meant any gene that encodes a ClpP protease.

By "ClpP activity" is meant enzymatic activity of Clp protease, as evidenced by cleavage of a Clp protease substrate, for example, a misfolded protein, RpoS, lO protein, and Mu vir repressor. ClpP activity may directly measured by measuring Clp enzymatic activity. ClpP activity also may be determined by measuring *clpP* mRNA levels or ClpP polypeptide levels, which

reflect relative changes in: transcription of a *clpP* gene, post-transcriptional degradation of a *clpP* mRNA, translation of a *clpP* mRNA, or post-translational degradation of a ClpP polypeptide. In all cases, a change in ClpP activity in a sample, for example, a sample exposed to an environmental stimulus such as a change in nutrient status or the addition of a chemical, is measured by an increase or decrease of at least 30%, more preferably at least 40%, still more preferably at least 55%, and most preferably by at least 70%, relative to a sample not exposed to the environmental stimulus.

By "non-*E. coli* ClpP" or "non-*E. coli clpP*" is meant a ClpP polypeptide or nucleic acid that is not the ClpP polypeptide or nucleic acid that is naturally encoded by the endogenous *E. coli* genome.

By "homologue" is meant a gene (e.g., a gene encoding a polypeptide component of pili or flagella, or a polypeptide that regulates synthesis or function of pili or flagella) whose nucleic acid hybridizes at low stringency to the nucleic acid of a reference gene, and whose encoded polypeptide displays a biological activity similar to that of the polypeptide encoded by the reference gene. For example, the *Vibrio paramaemolyticus flaE*, *Salmonella typhimurium flgK*, and *P. fluorescens sad-14* genes are homologues of one another. The effect of a homologue on synthesis of pili or flagella may be assessed by measuring mRNA or polypeptide levels of pilus or flagellum components. Function of pili or flagella may be measured by motility assays, such as those known in the art and described herein.

By "biological activity" is meant an activity associated with biofilm formation, as provided herein below.

By "high stringency conditions" is meant conditions that allow hybridization comparable with the hybridization that occurs during an overnight incubation using a DNA probe of at least 500 nucleotides in length,

in a solution containing 0.5 M $NaHPO_4$, pH 7.2, 7% SDS, 1 mM EDTA, 1% BSA (fraction V), and 100 μg/ml denatured, sheared salmon sperm DNA, at a temperature of 65° C, or a solution containing 48% formamide, 4.8X SSC (150 mM NaCl, 15 mM trisodium citrate), 0.2 M Tris-Cl, pH 7.6, 1X Denhardt's solution, 10% dextran sulfate, 0.1% SDS, and 100 μg/ml denatured, sheared salmon sperm DNA, at a temperature of 42° C (these are typical conditions for high stringency Northern or Southern, or colony hybridizations). High stringency hybridization may be used for techniques such as high stringency PCR, DNA sequencing, single strand conformational polymorphism analysis, and *in situ* hybridization. The immediately aforementioned techniques are usually performed with relatively short probes (e.g., usually 16 nucleotides or longer for PCR or sequencing, and 40 nucleotides or longer for *in situ* hybridization). The high stringency conditions used in these techniques are well known to those skilled in the art of molecular biology, and may be found, for example, in F. Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 1997, hereby incorporated by reference.

By "low stringency" is meant conditions that allow hybridization comparable with the hybridization that occurs during an overnight incubation at 37°C using a DNA probe of at least 500 nucleotides in length, in a solution containing 20% formamide, 5X SSC, 50 mM sodium phosphate (pH 7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured, sheared salmon sperm DNA (these are typical conditions for low stringency Northern, Southern, or colony hybridizations). Low stringency hybridization may be used for techniques such as low stringency PCR, which is usually performed with relatively short probes (e.g., usually 16 nucleotides). Factors that alter hybridization stringency (e.g., the relative likelihood of forming a duplex

between a single stranded probe and a target nucleic acid) are well known in the art, and are described in Ausubel, *supra*, at pages 2.10.8-2.10.16.

By "probe" or "primer" is meant a single-stranded DNA or RNA molecule of defined sequence that can base-pair to a second DNA or RNA molecule that contains a complementary sequence (the "target"). The stability of the resulting hybrid depends upon the extent of the base-pairing that occurs. The extent of base-pairing is affected by parameters such as the degree of complementarity between the probe and target molecules and the degree of stringency of the hybridization conditions. The degree of hybridization stringency is affected by parameters such as temperature, salt concentration, and the concentration of organic molecules such as formamide, and is determined by methods known to one skilled in the art. Probes or primers specific for nucleic acid encoding a *sad* gene preferably have at least 40% sequence identity, more preferably at least 45-55% sequence identity, even more preferably at least 60-75% sequence identity, still more preferably at least 80-90% sequence identity, and most preferably 100% sequence identity. Probes may be detectably-labelled, either radioactively, or non-radioactively, by methods well-known to those skilled in the art. Probes are used for methods involving nucleic acid hybridization, such as: nucleic acid sequencing, nucleic acid amplification by the polymerase chain reaction, single stranded conformational polymorphism (SSCP) analysis, restriction fragment polymorphism (RFLP) analysis, Southern hybridization, Northern hybridization, *in situ* hybridization, and electrophoretic mobility shift assay (EMSA).

By "identity" is meant that a polypeptide or nucleic acid sequence possesses the same amino acid or nucleotide residue at a given position, compared to a reference polypeptide or nucleic acid sequence to which the first

sequence is aligned. Sequence identity is typically measured using sequence analysis software with the default parameters specified therein, such as the introduction of gaps to achieve an optimal alignment (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705).

By "substantially identical" is meant a polypeptide or nucleic acid exhibiting, over its entire length, at least 40%, preferably at least 50- 85%, more preferably at least 90%, and most preferably at least 95% identity to a reference amino acid or nucleic acid sequence. For polypeptides, the length of comparison sequences is at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably at least 35 amino acids. For nucleic acids, the length of comparison sequences is at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably at least 110 nucleotides.

By "substantially pure polypeptide" is meant a polypeptide (or a fragment thereof) that has been separated from the components that naturally accompany it. Typically, the polypeptide is substantially pure when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the polypeptide is a *sad* polypeptide that is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, pure. A substantially pure *sad* polypeptide may be obtained, for example, by extraction from a natural source (e.g., a bacterium), by expression of a recombinant nucleic acid encoding a *sad* polypeptide, or by chemically synthesizing the polypeptide. Purity can be measured by any appropriate method, e.g., by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

A protein is substantially free of naturally associated components when it is separated from those contaminants that accompany it in its natural state. Thus, a protein that is chemically synthesized or produced in a cellular system different from the cell from which it naturally originates will be substantially free from its naturally associated components. Accordingly, substantially pure polypeptides are not only those derived from the organisms in which they naturally occur, but also those synthesized in organisms genetically engineered to express a given polypeptide.

By "substantially pure DNA" is meant DNA that is free of the genes which, in the naturally-occurring genome of the organism from which the DNA of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA that is part of a hybrid gene encoding additional polypeptide sequence.

By "transformation" is meant any method for introducing foreign molecules into a cell (e.g., a bacterial, yeast, fungal, algal, plant, or animal cell). Lipofection, DEAE-dextran-mediated transfection, microinjection, protoplast fusion, calcium phosphate precipitation, transduction (e.g., bacteriophage, adenoviral or retroviral delivery), electroporation, and biolistic transformation are just a few of the methods known to those skilled in the art which may be used.

By "transformed cell" is meant a cell (or a descendent of a cell) into which a DNA molecule encoding a polypeptide of the invention has been introduced, by means of recombinant DNA techniques.

By "promoter" is meant a minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell type, developmental status, and nutrient status, or inducible by external signals or agents; such elements may be located in the 5' or 3' or internal regions of the native gene.

By "operably linked" is meant that a gene and one or more regulatory sequences are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequences.

By "detectably-labeled" is meant any means for marking and identifying the presence of a molecule, e.g., an oligonucleotide probe or primer, a gene or fragment thereof, a cDNA molecule, or an antibody. Methods for detectably-labeling a molecule are well known in the art and include, without limitation, radioactive labeling (e.g., with an isotope such as ^{32}P or ^{35}S) and nonradioactive labeling (e.g., chemiluminescent labeling, or fluorescent labeling, e.g., with fluorescein).

By "sample" is meant a specimen containing bacterial cells, cell lysates, cell extracts, or mixtures of partially- or fully purified molecules, such as polypeptides or nucleic acids. Samples may be purified or fractionated by methods known in the art, including, but not limited to, differential precipitation or centrifugation, column chromatography, and gel electrophoresis.

By "specifically binds" is meant that an antibody recognizes and binds a given *sad* polypeptide but that does not substantially recognize and bind other molecules in a sample, e.g., a biological sample, that naturally includes

protein. Preferred samples include bacterial cells and cell lysates or cell extracts, including partially purified cell extracts.

By "expose" is meant to allow contact between an animal, cell (prokaryotic or eukaryotic), lysate or extract derived from a cell, or molecule derived from a cell, and a test compound, nutrient (such as citrate), or ion (such as Fe^{2+} or Fe^{3+}).

By "test compound" is meant a chemical, be it naturally-occurring or artificially-derived, that is surveyed for its ability to modulate biofilm formation, by employing one of the assay methods described herein. Test compounds may include, for example, peptides, polypeptides, synthesized organic molecules, naturally occurring organic molecules, nucleic acid molecules, and components thereof.

By "assaying" is meant analyzing the effect of a treatment or exposure, be it chemical or physical, administered to cells (e.g., bacterial cells) that are capable of forming biofilms. The material being analyzed may be a cell, a lysate or extract derived from a cell, or a molecule derived from a cell. The analysis may be, for example, for the purpose of detecting altered gene expression, altered nucleic acid stability (e.g. mRNA stability), altered protein stability, altered protein levels, or altered protein biological activity. The means for analyzing may include, for example, nucleic acid amplification techniques, reporter gene assays, antibody labeling, immunoprecipitation, enzymatic assays, measurement of the presence and/or function of physical structures such as flagella or pili (e.g., by motility assays such as swarming or twitching motility assays), measurement of biofilm formation, such as measurement of crystal violet (CV) staining or cell attachment, as described herein, and by other techniques known in the art for conducting the analysis of the invention.

By "modulating" is meant changing, either by decrease or increase.

By "a decrease" is meant a lowering in the level of: a) protein, e.g., measured by ELISA; b) reporter gene activity, e.g., measured by reporter gene assay, for example, lacZ/β-galactosidase, green fluorescent protein, luciferase, etc.; c) mRNA levels, e.g., measured by PCR relative to an internal control, for example, a "housekeeping" gene product such as ribosonal RNA; d) biofilm formation, e.g., as measured by crystal violet staining or counting attached cells; e) enzymatic activity of a polypeptide involved in biofilm formation, e.g., enzymatic activity of ClpP; or f) measurement of flagella or pilus function, e.g., by motility assays. In all cases, the lowering is preferably by at least 30%, more preferably by at least 40%, and even more preferably by at least 100%.

By "an increase" is meant a rise in the level of: a) protein, e.g., measured by ELISA; b) reporter gene activity, e.g., measured by reporter gene assay, for example, lacZ/β-galactosidase, green fluorescent protein, luciferase, etc.; c) mRNA levels, e.g., measured by PCR relative to an internal control, for example, a "housekeeping" gene product such as ribosonal RNA; d) biofilm formation, e.g., as measured by crystal violet staining or counting attached cells; e) enzymatic activity of a polypeptide involved in biofilm formation, e.g., enzymatic activity of ClpP; or f) measurement of flagella or pilus function, e.g., by motility assays. In all cases, the rise is preferably by at least 50%, more preferably by at least 80%, and even more prefcrably by at least 95%.

By "protein" or "polypeptide" or "polypeptide fragment" is meant any chain of more than two amino acids, regardless of post-translational modification (e.g., glycosylation or phosphorylation), constituting all or part of a naturally-occurring polypeptide or peptide, or constituting a non-naturally occurring polypeptide or peptide.

By "consecutive" is meant that a series of nucleotides exists as an unbroken sequence, i.e., uninterrupted by other nucleotides.

Brief Description of the Drawings

Fig. 1 is a representation of a photograph showing a biofilm formed by wild-type *P. fluorescens* and a graph showing quantitation of biofilm formation over time.

Fig. 2 is a graph demonstrating that protein synthesis is required for biofilm formation by *P. fluorescens*.

Fig. 3 is a representation of a photograph showing that biofilms are not formed by *P. fluorescens sad* mutants.

Fig. 4 (A-D) is a series of graphs showing biofilm formation on various surfaces by wild-type *P. fluorescens* and *sad* mutants.

Fig. 5 is a graph showing restoration of biofilm formation in a *clpP* mutant complemented with *clpP*$^+$ (wild-type clpP).

Fig. 6 is a representation of two phase-contrast photomicrographs showing restoration of biofilm formation in a *clpP* mutant complemented with *clpP*$^+$ (wild-type clpP).

Fig. 7 is a graph showing nutrient-mediated rescue of the biofilm formation defect in *P. fluorescens sad* mutants.

Fig. 8 is a diagram depicting a genetic model for biofilm formation in *P. fluorescens*.

Fig. 9 is a series of *sad* gene identifier sequences.

Fig. 10 is a representation of a photograph showing that nutrients affect biofilm formation in *E. coli*.

Fig. 11 is a representation of a photograph showing biofilm formation by wild-type and mutant *E. coli* strains.

Fig. 12 is a graph that shows quantification of biofilm formation in various *E. coli* strains.

Figs. 13A-13D are representation of photomicrographs showing biofilm formation by wild-type and mutant *E. coli* strains.

Fig. 14 is a graph showing inhibition of biofilm formation by α-methyl-D-mannoside.

Fig. 15 is a diagram showing a model for initiation of *E. coli* biofilm formation.

Fig. 16 is a representation of a photograph showing biofilm formation phenotypes in wild-type and mutant *P. aeruginosa* strains.

Fig. 17 is a representation of a photograph of a motility assay of wild-type and mutant *P. aeruginosa* strains.

Fig. 18 is a representation of a photograph of a twitching motility assay of wild-type and mutant *P. aeruginosa* strains.

Fig. 19 is a representation of a photomicrograph showing the edge morphology of wild-type and mutant *P. aeruginosa* colonies.

Fig. 20 is a representation of a series of phase-contrast photomicrographs showing a timecourse of biofilm formation by wild-type *P. aeruginosa*.

Fig. 21 is a representation of a series of phase-contrast photomicrographs showing biofilms formed by wild-type and mutant *P. aeruginosa* at 3 hours and 8 hours after biofilm initiation.

Figs. 22A-22I are representations of phase-contrast photomicrographs that show the role of twitching motility in biofilm formation by wild-type *P. aeruginosa*.

Fig. 23 is a schematic diagram of a model for the role of flagella and type IV pili in biofilm formation by *P. aeruginosa*.

Description of the Preferred Embodiments

To dissect the complex phenomenon of biofilm formation, we employed a genetic approach to identify genes required for the early stages of biofilm formation. We found that pili are essential for initial attachment to abiotic surfaces, and that flagella are necessary for biofilm spreading upon such surfaces. In addition, motility, but not chemotaxis, is crucial during the early biofilm formation. We observed that protein synthesis is necessary for initiation of biofilm formation; in contrast, we noted that high osmolarity inhibits biofilm formation.

Our genetic screen in *P. fluorescens* identified *sad* genes whose products are involved in flagellar synthesis and function, and a *sad* gene whose product displays sequence homology to the *E. coli* ClpP protein, a component of the Clp protease.

Our findings indicate the existence of at least two genetic pathways involved in biofilm formation, and suggest that cells, in response to environmental signals, can adopt multiple strategies for initiating cell-to-surface interactions.

Biofilm formation in *P. fluorescens*

The experiments described herein show that: (a) *P. fluorescens* can form biofilms on an abiotic surface under a range of growth conditions; (b) protein synthesis is required for the earliest events of biofilm formation, suggesting that biofilm formation is a regulated process in this organism; (c) one (or more) extra-cytoplasmic proteins plays a role in interactions with an abiotic surface, and that the surface-exposed protein(s) may constitute the adhesion that mediates direct cell-to-surface contact; and (d) the osmolarity of the medium can impact the ability of *P. fluorescens* to form biofilms.

Mutants of *P. fluorescens* defective for the initiation of biofilm formation on an abiotic surface (PVC) were isolated and designated *s*urface *a*ttachment *d*efective (*sad*). In addition to defects in forming biofilms on PVC, the *sad* mutants were also unable to initiate biofilm formation on other hydrophobic and hydrophilic surfaces. These data suggest that mutants identified on a single surface (i.e., PVC) may have defects in attachment on a wide range of abiotic (and potentially biotic) surfaces.

The initial search for mutants defective in biofilm formation was performed on minimal medium supplemented with glucose and CAA. However, approximately half of the *sad* mutants could be rescued for their biofilm formation defects (including the non-motile strains and the *clpP* mutant; see below) by supplementing the minimal glucose/CAA medium with iron, or by growing the strains with minimal medium supplemented with citrate or glutamate as the sole source of carbon and energy.

Not all nutrients that promote biofilm formation in the wild-type strain restore the ability of *sad* mutants to form a biofilm. For example, malate and mannitol allow growth and formation of biofilms in the wild type strain to levels comparable to glutamate- or citrate- grown cells, but do not rescue the biofilm formation defect of any of the *sad* mutants.

At this point it is not clear why glutamate and citrate, but not malate and mannitol, have the ability to rescue the biofilm formation defect of a subset of the *sad* mutants. *P. fluorescens* is a plant root colonizer, and it is possible that glutamate and/or citrate released by the plants may promote the formation of biofilms on the plant root. Consistent with this idea, recent studies have shown that citrate is the major organic acid found in exudates of roots and seedlings of tomato plants.

Iron was also found to rescue a subset of the biofilm formation mutants. In these experiments, iron was provided at 3 μM, a concentration of iron not normally seen in natural settings. Providing even 10-fold less iron in the medium (0.3 μM) results in loss of the rescue of the biofilm formation phenotype.

Rescue of some *sad* mutants by growth on citrate, glutamate, or in the presence of exogenous iron indicates that cells can form biofilms on an abiotic surface in the absence of flagella-mediated motility. As described below, flagella appear to play an important role in the ability of cells to form biofilms. However, in our system, under certain environmental conditions (i.e., cells grown on citrate, glutamate, or in the presence of excess exogenous iron) the flagellum appears dispensable for formation of *P. fluorescens* biofilms on PVC. It is possible that the cells use an alternative form of locomotion in the absence of a flagellum, such as twitching motility, but only do so in response to the appropriate environmental signals.

Our biofilm mutants strains contain disruptions in novel genes, genes involved in flagellar synthesis, and in a gene that shows sequence homology to the *E. coli* ClpP protein. This protein is a subunit of the *E. coli* cytoplasmic Clp protease (Gottesman, S. and Maurizi, M.R., 1992, *Microbiol. Rev.* 56: 592-621). Clp protease is involved in the degradation of misfolded proteins, RpoS, lO protein, and Mu vir repressor (Chung, C. H., et al., 1996, *Biol. Chem.* 377: 549-554; Damerau, K. and St. John, A.C., 1993, *J. Bacteriol.* 175: 53-63; Pratt, L. and Silhavy, T. J., 1996, *Proc. Natl. Acad. Sci . USA* 93: 2488-2492; Schweder, T., et al., 1996, *J. Bacteriol.* 178: 470-476).

Based on its known activities and on our results, it appears that ClpP is involved in the regulation of biofilm formation. The target protein(s) of ClpP required for the regulation of biofilm formation (as well as the signaling

pathway regulating Clp protease function) remain to be elucidated. Interestingly, ClpP does not appear to be required for biofilm formation under all growth conditions. The *clpP* mutant was first isolated in a screen for strains defective in biofilm formation in minimal glucose/CAA medium. However, ClpP function can be bypassed by growth on citrate, glutamate, or in the presence of exogenous iron.

We have found at least three overlapping pathways leading to the initiation of biofilm formation on an abiotic surface. One pathway (represented by 15 mutants) is functional on glucose/CAA medium independent of growth with citrate, glutamate, or exogenous iron. A second pathway, represented by *sad-19*, appears to be utilized by cells grown on minimal glucose/CAA, minimal glucose/CAA plus iron, and minimal glutamate, however, the defect in the strain carrying *sad-19* can be bypassed by growth on citrate. Twelve mutants, represented by the strain carrying allele *sad-18*, are not rescued for biofilm formation under any condition tested. These mutants may be defective for functions common to all of the biofilm formation pathways. The extent of the overlap among these pathways is unclear and will require further analyses. It is also possible that there are additional, as yet unidentified signals, which regulate biofilm formation.

Fig. 8 shows our current genetic model for the initiation of biofilm formation in *P. fluorescens*. We propose that multiple pathways can be utilized to initiate interactions with a surface, and that these pathways can be regulated by varying environmental parameters. Environmental signals may include carbon/energy sources and iron availability. Our genetic analyses indicate that there may be functions, such as those disrupted in the strain carrying allele *sad-18*, which are common to all known biofilm formation pathways. All of the mutants shown here, except for *fliP*, *flaE*, *sad-16*, *sad-20*, and *sad-22* are

motile. Our genetic analysis has begun to decipher the signaling pathways and structural genes which play a role in forming biofilms on an abiotic surface. It is still unclear which loci are required for sensing and responding to the signals required for biofilm formation (ClpP may play a role in this process) and which loci participate directly in the cell-to-surface interactions.

Of the 24 *P. fluorescens* mutants analyzed in this study and shown in Fig. 8, only 3 of the mutants had defects in genes of known function. These data suggest that we have isolated a number of new genes. Based on our molecular analyses of the DNA sequence flanking the transposon insertions, we know that the mutants are not siblings. However, it is possible that we have identified multiple mutations within a single gene or operon, a question that is currently under investigation.

Biofilm formation in *E. coli*

In addition to using *P. fluorescens* as an experimental model, we have used the well-studied and genetically tractable organism, *E. coli*, to rapidly identify genes required for the initial stages of biofilm formation.

As a result of our studies, we have made the surprising discovery that, under the conditions used in our experiments, chemotaxis is not required for the initiation of *E. coli* biofilm formation. In contrast, we conclude that motility is critical for normal biofilm formation; cells defective in flagellar biosynthesis or motility attach poorly to PVC, and the few cells that do attach are often located in small, dense clusters. The observation of small cell clusters in paralyzed or non-flagellated cell strains suggests that, in addition to enhancing initial surface contact, motility contributes to the initial spread of a biofilm by facilitating movement of cells along an abiotic surface.

Although it is possible that flagella also play a role in mediating actual adherence to abiotic surfaces, the fact that there is no phenotypic difference observed in the attachment (at the microscopic level) of paralyzed cells and non-flagellated cells to surfaces, compared to flagellated cells, does not support this hypothesis. Although flagella, motility, and/or chemotaxis have previously been implicated in biofilm formation in other organisms (DeFlaun, et al., *Appl. Environ. Microbiol.* 60:2637-2642, 1994; Graf, et al., *J. Bacteriol.*, 176:6986-6991, 1994; Korber, et al., *Appl. Environ, Microbiol.*, 60:1421-1429, 1994; Korber et al., *Pseudomonas fluorescens Microb. Ecol.*, 18:1-19. 1989; Lawrence, et al., *Microb. Ecol.*, 14:1-14, 1987; Mills and Powelson, *Bacterial Adhesion:Molecular and Ecological Diversity*, John Wiley & Sons, Inc., New York, Vol. pp. 25-57, 1996), these studies did not provide molecular characterization of the strains; therefore, the possibility that these strains contained pleiotropic defects could not be ruled out.

Moreover, prior to our molecular descriptions of the lesions conferring biofilm defects, it has been difficult to clearly define potential roles (adherence, motility, and/or chemotaxis) for flagella in biofilm development. For example, one could envision flagella functioning in three non-mutually exclusive roles: (1) flagellar-mediated chemotaxis might enable planktonic cells to swim towards nutrients associated with a surface or towards signals generated by cells attached to an abiotic surface, (2) flagellar-mediated motility might be required to overcome repulsive forces at a surface, enabling bacteria to initially reach a surface, and/or (3) flagella might function in a direct fashion by physically adhering to an abiotic surface.

Our studies show that, in contrast to flagella, type I pili are essential for initial attachment of *E. coli* prior to biofilm formation: cells harboring lesions in genes encoding proteins involved in the regulation or biogenesis of

type I pili do not efficiently attach to the abiotic surfaces tested. Indeed, only rarely do cells lacking type I pili attach. Moreover, cells lacking pili never form clusters of adhered cells, as do paralyzed and non-flagellated cells that possess pili. In addition, we discovered that attachment is inhibited by the presence of mannose or α-methyl-D-mannose. Type I pili contain the mannose-specific adhesin, FimH, which plays a role in facilitating pathogenesis through specific interactions between FimH and mannose oligosaccharides present on eukaryotic cell surfaces. The observation that FimH is also critical for attachment to abiotic surfaces was surprising and leads us to assign a novel role to type I pili.

There are two simple models to explain how FimH functions to attach to abiotic surfaces. First, FimH may play an indirect role, binding to sugars and/or proteins associated with the abiotic surface. Although this is a formal possibility, this model would predict that small amounts of mannose might interact with the surface and function to stimulate attachment. However, the observation that even the smallest amount of mannose added inhibited attachment argues against this hypothesis. Alternatively, it is possible that the interaction is direct and involves a region of FimH involved in non-specific binding to abiotic surfaces. If this is the case, then the binding of mannose to FimH may somehow alter its conformation, masking the FimH region that interacts with abiotic surfaces.

The mannose-dependent inhibition of *E. coli* biofilm formation on abiotic surfaces may have general applications to other biofilm-forming bacteria. Bacteria that form biofilms on surfaces in medically and/or industrially relevant environments may also require the integrity of adhesions analogous to the requirement of *E. coli* for FimH. Thus, it is possible that the

formation of problematic biofilms could be blocked through treatment with innocuous materials such as mannose.

The observations described here lead to the following model to describe the initiation of *E. coli* biofilm formation. Motility, but not chemotaxis, enhances cells' initial contact with an abiotic surface. This requirement may reflect a necessity to overcome repulsive forces present at an abiotic surface to be colonized. Once a surface is reached, type I pili are required to achieve stable cell-to-surface attachment. The presence of the FimH adhesion, when it is not bound to mannose, promotes such stable adherence to abiotic surfaces. Finally, we hypothesize that motility facilitates the development of a mature biofilm by allowing movement along a surface, thereby promoting spread of the biofilm.

In the work described herein, the alleles isolated affect factors required for flagellar biogenesis, motility, and the regulation and biogenesis of type I pili. It is well established that flagellar-mediated motility and the ability to produce a number of pili contribute to the virulence of pathogenic bacteria. This leaves us with the suggestive overlap of functions essential for both biofilm formation and functions needed for pathogenicity. In this regard, screens such as the one described here may prove useful in the identification of gene products important for the pathogenicity of a variety of bacteria. In addition, the work with *E. coli* may serve as a paradigm for the study of bacteria less amenable to genetic and molecular approaches. Although we predict extensive similarities in the molecular mechanisms utilized by other biofilm-forming bacteria, distinguishing details will no doubt arise. Such distinctions should be especially informative as to the particular mechanisms utilized by bacteria that live in various environmental niches.

Biofilm formation in *P. aeruginosa*

We have isolated a non-motile strain of *P. aeruginosa* (containing an insertion mutation in a *flgK* homolog) that is unable to form a biofilm. This finding shows that flagella, motility, and/or chemotaxis are required for *P. aeruginosa* biofilm development. It is noteworthy that the *flgK* mutant of *P. aeruginosa* displays a phenotype that differs from the *E. coli* flagellar mutants. Specifically, the *flgK P. aeruginosa* strain has only a few cells that attach to PVC and no micro-colonies are formed. This highlights the point that despite a clear conservation (between *E. coli* and *P. aeruginosa*) in the use of flagella during biofilm development, the aspect(s) of flagellar structure and function utilized appear to be different.

In addition, we have found insertion mutations in genes required for functional type IV pili, which interfere with normal *P. aeruginosa* biofilm formation. *P. aeruginosa* strains lacking type IV pili form monolayers of cells attached to PVC, but do not proceed past this stage, i.e., do not form micro-colonies or multi-layered biofilms.

The above findings suggest that similar surface structures, such as pili and flagella, are important in both *E. coli* and *P. aeruginosa* for normal biofilm development. However, the precise functions of these structures, although perhaps overlapping, are not completely conserved between these species.

Screens for compounds that affect biofilm formation

Compounds that modulate biofilm formation have various medical, industrial, agricultural, and public works uses. For example, compounds that stimulate biofilm formation could be used to improve colonization of plant roots by beneficial bacteria. Conversely, compounds that inhibit biofilm

formation could be employed to restrict growth of bacteria on contact lenses, medical implants (e.g., artificial hips), walls of catheters, water and sewerage pipes, and within the lungs of infected patients.

The invention provides screens for the isolation of such useful biofilm-modulating compounds. For instance, the biofilm formation assays described in Examples I-IV below may be used to measure the effect of test compounds on biofilm formation, relative to biofilm formation in untreated control samples. High-throughput screens may also be readily performed.

Furthermore, the effect of test compounds on biofilm formation may be indirectly assessed by measuring their effect on *sad* biological activity (e.g., transcription of a *sad* gene or *sad*/reporter gene; post-transcriptional degradation or translation of a *sad* mRNA or *sad*/reporter mRNA; or post-translational degradation, enzymatic function, or structural function of a *sad* polypeptide or *sad*/reporter polypeptide) in treated vs. untreated samples, using enzymatic, ELISA, PCR, and reporter gene assays described herein and/or known in the art.

The effect of test compounds on biofilm formation may also be assessed by measuring their influence on pilus or flagellum synthesis, structure, or function, e.g., using ELISA, PCR, and reporter gene assays, or the various motility assays described below, all of which are well known to skilled artisans.

a) ELISA for the detection of compounds that modulate biofilm formation

Enzyme-linked immunosorbant assays (ELISAs) are easily incorporated into high-throughput screens designed to test large numbers of compounds for their ability to modulate levels of a given protein. When used in the methods of the invention, changes in the level of a *sad* protein in a sample,

relative to a control, reflect changes in the biofilm formation status of the cells within the sample. Protocols for ELISA may be found, for example, in Ausubel et al.,*Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 1997. Samples, such as lysates from bacterial cells treated with potential biofilm formation modulators, are prepared (see, for example, Ausubel et al., *supra*), and are loaded onto the wells of microtiter plates coated with "capture" antibodies against one of the *sad* proteins. Unbound antigen is washed out, and a *sad* protein-specific antibody, coupled to an agent to allow for detection, is added. Agents allowing detection include alkaline phosphatase (which can be detected following addition of colorimetric substrates such as *p*-nitrophenolphosphate), horseradish peroxidase (which can be detected by chemiluminescent substrates such as ECL, commercially available from Amersham, Malvern, PA) or fluorescent compounds, such as FITC (which can be detected by fluorescence polarization or time-resolved fluorescence). The amount of antibody binding, and hence the level of a *sad* protein within a lysate sample, is easily quantitated on a microtiter plate reader.

As a baseline control for *sad* protein levels in untreated cells, a sample from untreated cells is included. Ribosonal proteins may be used as internal standards for absolute protein levels, since their levels do not change over the preferred timecourse (e.g., 0 to 10 hours for a standard biofilm assay, or 0 to 30 minutes for a rapid biofilm assay, as described in the examples below). Alternatively, bacteria or bacterial cell lysate may be directly exposed to a compound in the absence of biofilm assay conditions. A positive assay result, for example, identification of a compound that decreases biofilm formation, is indicated by a decrease in *sad* protein levels, relative to *sad* protein levels observed in untreated cells that are allowed to form a biofilm.

Conversely, an increase in *sad* protein levels, relative to *sad* protein levels in untreated cells, indicates a compound that increases biofilm formation.

b) Reporter gene assays for compounds that modulate biofilm formation

Assays employing the detection of reporter gene products are extremely sensitive and readily amenable to automation, hence making them ideal for the design of high-throughput screens. Assays for reporter genes may employ, for example, colorimetric, chemiluminescent, or fluorometric detection of reporter gene products. Many varieties of plasmid and viral vectors containing reporter gene cassettes are easily obtained. Such vectors contain cassettes encoding reporter genes such as lacZ/β-galactosidase, green fluorescent protein, and luciferase, among others. We have constructed strains containing *sad* mutations described herein with lacZ fusions that may be used in such screens. Cloned DNA fragments encoding transcriptional control regions of interest are easily inserted, by DNA subcloning, into such reporter vectors, thereby placing a vector-encoded reporter gene under the transcriptional control of any gene promoter of interest. The transcriptional activity of a *sad* gene promoter operably linked to a reporter gene can then be directly observed and quantitated as a function of reporter gene activity in a reporter gene assay.

Bacteria containing one or more *sad*/reporter gene constructs are cultured under the appropriate conditions, e.g., under conditions that promote biofilm formation in a screen for a compound that inhibits biofilm formation. Alternatively, bacteria or bacterial cell lysates may be directly exposed to a compound in the absence of biofilm assay conditions. Compounds to be tested for their effect on biofilm formation are added to the bacteria. At appropriate timepoints, bacteria are lysed and subjected to the appropriate reporter assays,

for example, a colorimetric or chemiluminescent enzymatic assay for lacZ/β-galactosidase activity, or fluorescent detection of GFP. Changes in reporter gene activity of samples treated with test compounds, relative to reporter gene activity of appropriate untreated control samples indicate the presence of a compound that modulates biofilm formation.

In one embodiment, one construct could comprise a reporter gene such as lacZ or chloramphenicol acetyltransferase (CAT), operatively linked to a promoter from a *sad* gene. *Sad*/reporter gene constructs may be present within the genomic DNA of a bacterial cell to be tested, or may be present as an episomal DNA molecule, such as a plasmid. A second reporter gene operably linked to a second promoter is included as an internal control. This could be an episomal reporter gene operatively linked, for example, to a glucose phosphotransferase or phosphofructokinase gene. The glucose phosphotransferase or phosphofructokinase gene is expressed in bacteria growing on glucose. The amount of activity resulting from an internal control reporter gene that is operably linked to a glucose kinase (or analogous) promoter will indicate the proportion of live growing cells within a treated sample, relative to an untreated sample. The *sad* reporter gene activity is normalized to the internal control reporter gene activity. As a result of the normalization, a relative decrease in *sad* promoter activity indicates a compound that modulates biofilm formation by down-regulating *sad* gene transcription (rather than, e.g., a compound that inhibits cell growth or kills cells, thus giving the appearance of decreased *sad* gene transcription).

c) Quantitative PCR of sad mRNA as an assay for compounds that modulate biofilm formation

The polymerase chain reaction (PCR), when coupled to a preceding reverse transcription step (rtPCR), is a commonly used method for detecting vanishingly small quantities of a target mRNA. When performed within the linear range, with an appropriate internal control target (employing, for example, a housekeeping gene such as the glucose phosphotransferase or phosphofructokinase), such quantitative PCR provides an extremely precise and sensitive means for detecting slight modulations in mRNA levels. Moreover, this assay is easily performed in a 96-well format, and hence is easily incorporated into a high-throughput screening assay. Bacterial cells are cultured under the appropriate biofilm-inducing or -inhibiting conditions, in the presence or absence of test compounds. The cells are then lysed, the mRNA is reverse-transcribed, and the PCR is performed according to commonly used methods (such as those described in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 1997), using oligonucleotide primers that specifically hybridize with the nucleic acid of interest. In one embodiment, the target mRNA could be that of one or more of the *sad* genes. Analogously to the *sad* protein result described above, changes in product levels of samples exposed to test compounds, relative to control samples, indicate test compounds with biofilm formation-modulating activity.

d) Test Compounds

In general, novel compounds for modulating biofilm formation are identified from large libraries of both natural product or synthetic (or semi-synthetic) extracts or chemical libraries according to methods known in the art. Those skilled in the field of chemical discovery and development will

understand that the precise source of test extracts or compounds is not critical to the screening procedure(s) of the invention. Accordingly, virtually any number of chemical extracts or compounds can be screened using the exemplary methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as modification of existing compounds. Numerous methods are also available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and nucleic acid-based compounds. Synthetic compound libraries are commercially available from Brandon Associates (Merrimack, NH) and Aldrich Chemical (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceangraphics Institute (Ft. Pierce, FL), and PharmaMar, U.S.A. (Cambridge, MA). In addition, natural and synthetically produced libraries are produced, if desired, according to methods known in the art, e.g., by standard extraction and fractionation methods. Furthermore, if desired, any library or compound is readily modified using standard chemical, physical, or biochemical methods.

In addition, those skilled in the art of chemical discovery and development readily understand that methods for dereplication (e.g., taxonomic dereplication, biological dereplication, and chemical dereplication, or any combination thereof) or the elimination of replicates or repeats of materials already known for their effects on biofilm formation should be employed whenever possible.

When a crude extract is found to modulate biofilm formation, further fractionation of the positive lead extract is necessary to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude extract having an effect on biofilm formation. The same assays described herein for the detection of activities in mixtures of compounds can be used to purify the active component and to test derivatives thereof. Methods of fractionation and purification of such heterogenous extracts are known in the art. If desired, compounds shown to be useful agents for treatment are chemically modified according to methods known in the art. Compounds identified as being of medical or industrial value may be subsequently analyzed using the appropriate biofilm formation model.

Below are examples of high-throughput systems useful for evaluating the efficacy of a molecule or compound in stimulating or inhibiting biofilm formation.

e) Uses

Compounds identified using any of the methods disclosed herein may be administered to patients or experimental animals, applied to the fluid-contacting surfaces of medical devices, such as catheter lines, contact lenses, and surgical implants, applied to the fluid-contacting surfaces of industrial devices, such as pipes, or applied to soil, seeds, or plant roots by methods known in the various medical, manufacturing, and agricultural arts. Moreover, fluid-contacting surfaces may be impregnated with the compounds of the invention.

The following examples are meant to illustrate, not limit, the invention.

Example I: General Methods

Bacterial strains, media, and chemicals.

All *P. fluorescens* strains and plasmids used in the experiments described in Example II below are shown in Table 1. *P. fluorescens* strain WCS365 and *P. aeruginosa* strain PA14 were grown at 30°C and 37°C, respectively, on rich medium (Luria Bertani; LB) or minimal medium, unless otherwise noted. The minimal medium used was M63 (Pardee, A.B., et al., 1959, *J. Mol. Biol.* 1: 165-178) supplemented with glucose (0.2%), $MgSO_4$ (1 mM) and, where indicated, casamino acids (CAA, 0.5%), citric acid (0.4%), glutamic acid (monosodium salt, 0.4%) or $FeSo_4 \cdot 7H_2O$ (3 μM). Unless otherwise indicated, all carbon sources were provided at 0.4%.

For the experiments described in Example III, W3110 (*E. coli* K12 F-l- IN(rrnD-rrnE)1 rph-1) was used as the parental strain; all strains described in Example III are either W3110 or derivatives of this strain. The media and growth conditions used have been previously described (Pardee, A.B. et al., *supra*; Silhavy, T. et al., *Experiments with gene fusions*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1984), and casamino acids were added at a concentration of 0.5%.

Whenever antibiotics were used, they were added at the following concentrations: *E. coli* : ampicillin (Ap), 150 μg/ml; naladixic acid (Nal), 20 μg/ml; tetracycline (Tc), 15 μg/ml; kanamycin (Kn), 50 μg/ml; *P. fluorescens*: Tc, 150 μg/ml; gentamycin (Gm), 100 μg/ml; Kn, 500 μg/ml; *P. aeruginosa*: Tc, 150 μg/ml. Pronase E was obtained from Sigma Chemical Co. (St. Louis, MO).

Table 1. Strains and Plasmids

Strain (Relevant genotype)	Reference
Pseudomonas fluorescens strain WCS365	(Geels and Schippers, *Phytopathol. Z.*, **108**:207-214, 1983); Simons, et al., *Mol. Plant Microbe Inter.*, **9**:600-607, 1996)
P. fluorescens clpP::Tn5-B30(Tc^r)	This study
P. fluorescens fliP::Tn5-B30(Tc^r)	This study
P. fluorescens flaE::Tn5-B30(Tc^r)	This study
P. fluorescens sad-10::Tn5-B30(Tc^r)	This study
P. fluorescens sad-16::Tn5-B30(Tc^r)	This study
P. fluorescens sad-18::Tn5-B30(Tc^r)	This study
P. fluorescens sad-19::Tn5-B30(Tc^r)	This study
P. fluorescens sad-20::Tn5-B30(Tc^r)	This study
P. fluorescens sad-21::Tn5-B30(Tc^r)	This study
P. fluorescens sad-22::Tn5-B30(Tc^r)	This study
ZK126 (*clpP*$^+$, E. coli W3110)	(Connell, et al., *Mol. Microbiol.*, **1**:195-204, 1987)
Plasmids	
pTn5-B22 (Gm^r, *'lacZ*)	(Simon, et al., *Gene*, **80**:160-169, 1989)
pTn5-B30 (Tc^r)	(Simon, et al., *Gene*, **80**:160-169, 1989)
pUC181.8 (Ap^r)	(Frank, et al., *J. Bacteriol.*, **1781**:5304-5313, 1989)
pSU39 (Kn^r)	(Martinez, et al., *Gene*, **68**:159-162, 1988)
pSMC26 (*clpP*$^+$, Kn^r, derivative of pSMC28)	This study
pSMC28 (derivative of pSU39, Kn^r, stably maintained in *Pseudomonas* spp.)	This study

Molecular and genetic techniques.

All plasmids were constructed in *E. coli* JM109 using standard protocols (Ausubel, F.A. et al., 1990, Current Protocols in Molecular Biology, Wiley Interscience, NY) then transferred to the appropriate strains by electroporation (Bloemberg, G.V. et al., 1997, *Appl. Environ. Microbiol.*, 63, 4543-4551)).

a) Transduction and transposon mutagenesis

Generalized transduction in *E. coli* using P1vir was performed as previously described (Silhavy, et al., *supra*). Genetic linkage analysis in *E. coli* was performed by using a P1vir lysate that had been grown on a pool of cells containing transposons randomly inserted throughout the chromosome (Kleckner, N., et al.,1991, *Methods in Enzymology*, 204, 139-180). Transductions into *P. aeruginosa* were performed as reported (Jensen, E.C. et al., 1998, *Appl. Environ. Microbiol.*, 64, 575-580).

Transposon mutants in *P. fluorescens* were generated using a modification of published protocols (Simons, M., et al., 1996, *Mol. Plant Microbe Inter.* 9: 600-607). Recipient (*P. fluorescens*) and donor (*E. coli* S17-1/pTn5::B30(Tc) or *E. coli* S17-1/pTn5::B322(Gm)) were grown in LB to late log phase (A600 = 0.6-0.8). After incubating *P. fluorescens* at 42°C for 15 min, 1 mL of the recipient was added to 0.25 mL of the donor in a 1.5 mL Eppendorf tube. The cells were pelleted in a microfuge, the medium decanted, and the cells resuspended in 50 µL of LB, and the entire 50 µL was spotted on an LB plate and incubated at 30°C for 24-48 hrs. After incubation, the cells were scraped from the LB plate and resuspended in 1 mL LB and 250 µL was subsequently plated on LB plates supplemented with Tc or Gm (to select for the Tn5 mutants) and Nal (to select against growth of the *E. coli* donor). The

resulting transposon mutants were screened for biofilm formation as described below.

Transposon mutants in *P. aeruginosa* were generated with Tn5-B30(Tcr) using a modification of published protocols (Simon, R.,1989, *Gene*, 80, 160-169) The resulting transposon mutants were screened for biofilm formation as described below.

b) PCR

The DNA sequence flanking transposon mutants was determined using arbitrary PCR (Caetano-Annoles, G., 1993, *PCR Methods Appl.* 3: 85-92). In this technique, the DNA flanking insertion sites is enriched in two rounds of amplification using primers specific to the ends of the Tn5 element and primers to random sequence that anneal to chromosomal sequences flanking the transposon.

PCR of P. fluorescens and P. aeruginosa transposon mutant DNA

In the first round, a primer unique to the right end of Tn5 elements (Tn5Ext, 5'-GAACGTTACCATGTTAGGAGGTC-3'; SEQ ID NO: 25) and arbitrary primer #1 (ARB1, 5'-GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT-3'; SEQ ID NO: 26) were used in 100 uL PCR reactions (1X Vent Polymerase buffer, $MgSO_4$ (1mM), dNTPs (0.25 mM), and Vent, exo-DNA polymerase (2 U) with 5 mL of an overnight LB-grown culture as the source of DNA. The first round reaction conditions were: i), 5 min. at 95°C ii) 6X (30 sec at 95°C, 30 sec at 30°C, 1 min 30 sec at 72°C), iii) 30X (30 sec at 95°C, 30 sec at 45°C, 2 min at 72°C).

Reactions for the second round of PCR were performed as described for the first round, except 5 μL of the first round PCR product was used as the source of DNA and the primers were ARB2 (5'-GGCCACGCGTCGACTAGTAC-3'; SEQ ID NO: 27) and Tn5Int (5'-CGGGAAAGGTTCCGTTCAGGACGC-3'; SEQ ID NO: 28). The ARB2 sequence is identical to the 5'-end of the ARB1 primer and the sequence of Tn5Int is identical to the right-most end of Tn5, near the junction between the transposon and the chromosome. The reaction conditions for the second round were 30X (30 sec at 95°C, 30 sec at 45°C, 2 min at 72°C).

PCR products were purified either from an agarose gel using β-agarase (NEB, Beverly, MA) or with the QIAquick Spin PCR Purification Kit (Qiagen Inc, Chatsworth, CA) as described by the manufacturer without modification. PCR products were sequenced using the Tn5Int primer at the Micro Core Facility, Department of Microbiology and Molecular Genetics, Harvard Medical School and compared to the Genbank DNA sequence database using the BLASTX program (Altschul, S.F., et al., 1990. *J. Mol. Biol.* 215: 403-410).

PCR of E. coli transposon mutant DNA

The first round of PCR reactions used the following primers: ARB1 (GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT; SEQ ID NO: 26) or ARB6 (GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC; SEQ ID NO: 29) and OUT1-L (CAGGCTCTCCCGTGGAG; SEQ ID NO: 30). The second round of PCR reactions used the following primers: ARB2 (GGCCACGCGTCGACTAGTAC; SEQ ID NO: 27) and PRIMER1L (CTGCCTCCCAGAGCCTG; SEQ ID NO: 31).

Following the second round of PCR amplification, PCR products were separated a 1.0% low melt agarose gels, and bands were excised from the gel. The agarose was digested with β-agarase, and the DNA was subjected to DNA sequence analysis utilizing PRIMER1L. Sequence analysis was carried out at the Biopolymers Laboratory of the Department of Biological Chemistry and Molecular Pharmacology of Harvard Medical School.

c) Southern blots

Southern blots were performed as follows: chromosomal DNA of the *sad* mutants was prepared (Pitcher, D.G., 1989, *Lett. Appl. Microbiol.*, 8, 151-156.), digested with EcoRI (Tn5-B30 does not have a EcoRI site), and transferred to GeneScreen Plus (NEN Research Products, Boston, MA) as reported (Ausubel, F.A. et al., 1990, Current Protocols in Molecular Biology. Wiley Interscience, NY). The hybridization was performed with the ECL direct nucleic acid labeling and detection system (Amersham Life Science, Buckinghamshire, England) following the manufacturer's instructions without modification. The DNA probe used was derived from the insertion sequence element (IS50) of Tn5 and generated using PCR with the Tn5 element as a template. The PCR primers used to generate the probe were IS50R.1 (5'-GCTTCCTTTAGCAGCCCTTGCGC-3'; SEQ ID NO: 32) and IS50R.2 (5'-CTTCCATGTGACCTCCTAACATGG-3'; SEQ ID NO: 33).

d) Cloning of integrated transposons

Selected transposons were cloned to determine additional DNA sequence flanking the transposon. Chromosomal DNA was prepared (Pitcher, D.G., et al.,1989. *Lett. Appl. Microbiol,* 8: 151-156), digested with *Eco*RI (there are no *Eco*RI sites in these Tn5 derivatives), and ligated with

pBluescript, KS+, Ap^r (Stratagene, La Jolla, CA) previously digested with *Eco*RI. Ligation mixes were electroporated into *E. coli* JM109, plated on LB supplemented with Ap, then printed onto LB supplemented with Ap (150 μg/ml) and Tc (10 μg/ml). The Ap^{r}Tc^r colonies were purified, plasmid DNA prepared, and the plasmids were sequenced with the Tn5Ext primer.

e) Construction of the *clpP*-carrying plasmid

A derivative of pSU39 (Martinez, E., et al., 1988, *Gene* 68: 159-162) was constructed that is stably maintained in *Pseudomonas* spp. The 1.8 kb *Pst*I "stabilizing fragment" of pUC181.8 (Frank, D.W., 1989, *J. Bacteriol.* 171: 5304-5313) was cloned into the *Pst*I site of pSU39, generating the plasmid pSMC28. The stabilizing fragment allows the stable replication of plasmids in *Pseudomonas* spp. To generate the plasmid required for complementation analysis, the *clpP* gene of *E. coli* (ZK126 W3110) was amplified with primers flanking *clpP* and also including the predicted promoter region of this gene. The PCR product was cloned into pSMC28, previously digested with *Hinc*II, generating plasmid pSMC26 (*clpP*$^+$).

Motility Assays.

Following strain construction involving alleles that affect flagella, motility, and/or chemotaxis, the presence (or absence) of flagella was confirmed using a simple staining procedure that has been previously described (Heimbrook, et al.,1989, *J. Clin. Microbiol.*, 27, 2612-2615). Motility and chemotaxis were analyzed using both swarm assays (Adler, J.,1966, *Science*, 153, 708-716.; Wolfe, A.J. and Berg, H.C.,1989, *Proc. Natl. Acad. Sci.* USA, 86, 6973-6977) and phase contrast microscopy of living cells. lNK1324 was used for insertion mutagenesis of W3110 as previously described (Kleckner, et

al., *supra*). Motility assays were performed on minimal agar plates (0.3%) supplemented with glucose and CAA and the distance that the cells migrated through the agar was determined after 16-24 hrs. Twitching motility was assessed as described (Whitchurch, C.B. et al., 1990, *Gene*, 101, 33-44).

Biofilm formation assay.

Our standard biofilm formation assay involves starting with relatively low number of cells (~10^6 CFU/ml) in minimal M63 medium supplemented with glucose and casamino acids (CAA) at 25°C to 37°C for 8 to 48 hours. Biofilm development can be monitored indirectly by following the increase in crystal violet (CV) staining over time; this purple dye stains the bacterial cells, but does not stain plastics such as polyvinylchloride (PVC). Alternatively, biofilm formation can be monitored with a rapid assay by starting with ~10^8 CFU/ml. In this way, biofilm formation can be detected after just 30 min. Using these assays, we tested the impact of various growth conditions and environmental signals on biofilm formation and searched for mutants defective in this process.

a) Screen for mutants defective in biofilm formation

This assay is based on the ability of bacteria to form biofilms on polyvinylchloride plastic (PVC), a material which is used to make catheter lines (Lopez-Lopez, G., et al., 1991, *J. Med. Microbiol.* 34: 349-353). Biofilm formation was assayed by the ability of cells to adhere to the wells of 96-well microtiter dishes made of PVC (Falcon 3911 Microtest III Flexible Assay Plate, Becton Dickinson Labware, Oxnard, CA) using a modification of a reported protocol (Fletcher, M., 1977, *Can. J. Microbiol.* 23: 1-6). The indicated medium (100 µL/well) was inoculated either from cells patched on LB agar

plates using a multi-prong device or a 1:100 dilution from an overnight LB culture. After inoculation, plates were incubated at 25°C to 37°C for 8-48 hours for *P. fluorescens* and *P. aeruginosa* or 10-24 hours for *E. coli*, then 25 µL of a 1% solution of CV was added to each well (this dye stains the cells, but not the PVC), the plates were incubated at room temperature for ~15 min, rinsed thoroughly and repeatedly with water, and scored for the formation of a biofilm. Fig. 1 shows the formation of the biofilm at the air-medium interface, monitored over a 10 hr period. Because of the growth conditions used in this assay (oxygen is the primary electron acceptor) *P. fluorescens* grows predominantly near the surface of medium in the microtiter wells. Crystal-violet-stained, surface-attached cells were quantified by solubilizing the dye in ethanol and determining the absorbance at 600 nm. The A_{600} values increased with time up to about 8-10 hours of incubation. Wells developed at 0 and 10 hours are shown above the graph in Fig. 1.

b) Rapid biofilm formation assay

To assess the formation of biofilms after 30 min instead of 10 hrs, *P. fluorescens* was grown overnight under conditions that only weakly stimulate biofilm formation (minimal glucose medium) resulting in a viable count of ~10^8 colony forming units (CFU)/ml. The planktonic cells were centrifuged, and resuspended in an equal volume of fresh minimal medium supplemented with glucose and CAA (conditions that stimulate biofilm formation) and assessed for biofilm formation using the CV-based assay described above. This method was used to assess the effects of the protein synthesis inhibitor Tc and protease treatment on biofilm formation.

c) Quantitation of biofilm formation

Biofilm formation was quantified by the addition of 2 X 200 µL of 95% ethanol to each CV-stained microtiter dish well, the ethanol was transferred to a 1.5 ml Eppendorf tube, the volume brought to 1 mL with dH_2O, and the absorbance determined at 540 nm in a spectrophotometer (DU-640 Spectrophotometer, Beckman Instruments Inc., Fullerton, CA). Alternatively, CV-stained biofilms were solubilized in 200 µL of 95% ethanol, of which 125 µL was transferred to a new polystyrene microtiter dish (Costar Corporation, Cambridge, MA), and the absorbance determined with a plate reader at 600 nm (Series 700, Microplate Reader, Cambridge Technology, Inc., Cambridge, MA). We also used these methods to quantify biofilm formation on polystyrene (Pro-bind Assay Plate, non-tissue culture treated, Becton Dickson & Co., Lincoln Park, NJ), polypropylene (1.5 mL microcentrifuge tube, Marsh Biomedical Products, Inc., Rochester, NY), and borosilicate glass (Kimax 51 culture tubes, VWR, S. Plainfield, NJ).

d) Microscopy

The visualization of *P. fluorescens* cells attached to PVC was performed as reported (Bloemberg, G.V., et al., 1997, *Microbiol.* 63: 4543-4551). Visualization of *P. aeruginosa* cells attached to PVC was performed by phase contrast microscopy (400X magnification) using a Nikon Diaphot 200 inverted microscope (Nikon Corp., Tokyo, Japan). The images were captured with a black and white CCD72 camera integrated with a Power Macintosh 8600/300 computer with video capability (Macintosh, Cupertino, CA). The images were processed with Scion Image software, a modification of NIH Image (NIH, Washington, DC) by the Scion Corporation (Frederick, MD).

e) Quantification of CV-stained attached *P. aeruginosa* cells and growth curves

Attached cells were quantified as described previously, with a few modifications (Genevaux, et al., 1996, *FEMS Microbiol. Lett.*, 142, 27-30; O'Toole, G.A. and Kolter, R., 1998, *Mol. Microbiol.*, 28:449-461). After wells had been stained with 125 ml of 1.0% CV, rinsed, and thoroughly dried, the CV was solubilized by the addition of 200 μL ethanol:acetone (80:20); or 95% ethanol (with no acetone). 80 μL of the solubilized CV was removed and added to a fresh polystyrene, 96-well dish, and OD_{600} or OD_{570} was determined using either a Series 700, Microplate Reader from Cambridge Technology, Inc. or an MR 700 Microplate Reader from Dynatech Laboratories, Inc.

Growth curves were determined by subculturing (1:100) the relevant strain into the appropriate medium and growing the culture at room temperature without shaking. OD_{600} readings were taken over time with a spectronic 20D+ from Spectronic Instruments, Inc.

Example II: Identification of mutations that affect biofilm formation in *Pseudomonas fluorescens*

Protein synthesis is required for biofilm formation.

There are marked differences in the profile of proteins synthesized by biofilm-grown cells versus planktonic cells. We hypothesized that *P. fluorescens* synthesizes proteins required to form biofilms in response to appropriate signals. One of the predictions of such a model is that protein synthesis inhibitors should block biofilm formation in an environment that would otherwise promote this process. To test this prediction, cells were incubated in the presence or absence of the protein synthesis inhibitor tetracycline (Tc, 150 µg/ml) in microtiter wells for 30 minutes, after which the

wells were developed with CV to assess biofilm formation using the rapid biofilm formation assay described above. As shown in Fig. 2, after 30 min, biofilm formation is strongly inhibited in the presence of 150 μg/ml Tc, compared to the untreated control (the extent of biofilm formation is expressed as the absorbance at 540 nm). This concentration of Tc does not reduce the numbers of viable planktonic cells (Tc-treated cultures, 1.0 x 10^8 CFU/ml; untreated control, 1.2 x 10^8 CFU/ml). This result indicates that new protein synthesis is required for *P. fluorescens* to form biofilms on an abiotic surface.

In contrast to the observation described above, continued protein synthesis is not required after the initial events of biofilm formation. Cells were first allowed to incubate in the microtiter wells for 30 minutes to form biofilms and then treated with Tc. After incubation for an additional 30 minutes in the presence of Tc, the microtiter dish wells were developed with CV to assess the extent of biofilm formation. There was no difference in biofilms (Fig. 2) or viable cell counts (not shown) between Tc-treated cells and untreated control cells.

These data suggest that the earliest events of biofilm development can be divided into two stages. The first stage, initial interaction with the abiotic surface, requires new protein synthesis. However, the subsequent stage (short-term maintenance of the attached cells) does not require synthesis of new proteins.

Extra-cytoplasmic proteins participate in biofilm formation.

Extra-cytoplasmic proteins, specifically those proteins on the surface of the bacterial cell, are thought to be important for bacterial attachment to abiotic substrates. To address the importance of such proteins in our biofilm system, we determined the effect of treatment with a protease, Pronase E

(added upon inoculation of the cells into microtiter wells) on the formation of biofilms, using the rapid attachment assay. The number of attached cells was markedly decreased in the wells treated with Pronase E (~5-10-fold) compared to untreated control wells. In contrast, the counts of viable planktonic cells were similar under both conditions (average viable counts for untreated samples, 1.5 x 10^8 CFU/ml and Pronase E treated samples, 2.4 x 10^8 CFU/ml), indicating that treatment with protease did not decrease the number of viable cells. This result indicates that at least one extra-cytoplasmic protein is necessary for adherence to PVC.

Environmental factors affect biofilm formation.

Because the nutritional content of the medium can regulate biofilm development, we tested various nutrients for their effects on the ability of *P. fluorescens* to form biofilms on PVC. The following additions to minimal M63-based media promoted the formation of biofilms: 0.2% glucose, 0.2% glucose + 0.5% CAA, 0.2% glucose + 3 μM $FeSO_4$, 0.5% CAA, 0.4% glutamate, 0.4% citrate, 0.4% malate, 0.4% mannitol, 0.4% xylose, and 0.4% glycerol. Although glucose alone does promote biofilm formation, the addition of iron or CAA stimulates biofilm formation by ~2- to 3-fold over glucose alone.

We assessed the effect of changes in osmolarity on the ability of *P. fluorescens* to form biofilms on PVC, using two osmolytes, NaCl and sucrose. The NaCl concentration was varied from 0 to 0.4 M in minimal medium supplemented with glucose and CAA. The growth of this strain was unaffected across this range of NaCl concentrations. However, at concentrations of NaCl at or above 0.2 M, biofilm formation was decreased by up to 4-fold, as assayed by CV staining. Cells grown in minimal medium as above, but supplemented

with 0 to 20% sucrose, also grew to equal final optical densities. At sucrose concentrations of 15% or 20%, however, biofilm formation decreased by greater than 4-fold when compared to control cultures lacking sucrose. It is important to note that the osmolarity of the medium used in these experiments with 0.2 M NaCl is approximately equal to medium supplemented with 15% (~0.44 M) sucrose. Taken together, these data strongly suggest that growth in high osmolarity (and not simply ionic strength) inhibits biofilm formation by *P. fluorescens* on PVC. Variations in the starting pH (from 5.0 to 8.5) of the growth medium had no effect on biofilm formation after incubation for 10 hrs under standard assay conditions.

The results presented above show that environmental conditions and the nutritional status of the medium can influence biofilm formation. Furthermore, as demonstrated by the experiments in which osmolarity was varied, there are environmental conditions that promote cell growth, but do not promote significant biofilm formation.

Isolation of mutants defective in biofilm formation.

To isolate strains defective in biofilm formation on an abiotic surface, Tn5-based transposons that confer Tc^r or Gm^r (Simon, R., et al., 1989, *Gene* 80: 160-169) were used to mutagenize *P. fluorescens*. Of the ~14,000 transposon mutants screened, 37 mutants (0.3%) were unable to form a biofilm (Fig. 3; assay was developed after a 10 hour incubation) and had a growth rate indistinguishable from the wild-type strain in liquid medium. These mutants were designated *s*urface *a*ttachment *d*efective (*sad*). Twenty-eight of these mutants (23 motile and 5 non-motile) were analyzed further. Fig. 4A shows the quantitation of the biofilm formed by representative *sad* mutants on PVC. As described below, various growth conditions rescue the biofilm formation defect

of some of the *sad* mutants. The subset of mutants presented in Fig. 4A-4D and in Table 2 was chosen to represent each of the phenotypic classes defined by the nutritional rescue experiments described below.

The biofilm formation screen described above was performed using microtiter dishes composed of PVC. However, it is clear that bacteria form biofilms on a wide range of abiotic surfaces. We tested the ability of wild type bacteria and selected mutants to form biofilms on relatively hydrophobic surfaces (PVC, polycarbonate, and polypropylene) and on a relatively hydrophilic surface (borosilicate glass). Wild type and mutant strains were allowed to form biofilms on these surfaces over a ten hour incubation period, then stained with CV and quantitated (Fig. 4A-4D). In general, mutants that were unable to form biofilms on PVC also were unable to form biofilms on the other surfaces tested, suggesting that a common genetic pathway is used to form biofilms on a range of abiotic surfaces. However, the strain carrying the *sad-10* allele is notable in that it has a biofilm formation defect on hydrophobic surfaces (PVC, polycarbonate, and polypropylene), but its biofilm formation phenotype on a hydrophilic surface (borosilicate glass) is indistinguishable from that of the wild type. In addition, the *sad-13* (*fliP*) mutant displayed a defect in biofilm formation on PVC, although this defect was less apparent on the other surfaces, especially polystyrene. In addition, the colony morphology of wild type bacteria vs. *sad* mutants was indistinguishable on LB medium.

Phenotypes of surface attachment defective mutants.

In order to further classify the *sad* mutants, they were assessed for the following phenotypes: growth in liquid medium, colony morphology, motility, fluorescent pigment production, biofilm formation under various

environmental conditions, and determination of their molecular defects. The results of the phenotypic (and molecular) analyses of a representative subset of the *sad* mutants is summarized in Table 2.

The growth rate of all of the *sad* mutants in minimal medium supplemented with glucose and CAA (standard assay conditions) was identical to the wild type. None of the mutants were auxotrophs as judged by growth on minimal medium supplemented only with glucose. All mutants were also tested for their growth rate on minimal glucose/CAA + 3 μM $FeSO_4$, minimal medium + citrate (0.4%), and minimal medium + glutamate (0.4%). Only those mutants whose growth rates were indistinguishable from the wild type growth rate under all growth conditions were analyzed further.

Motility is required for biofilm formation on biotic and abiotic surfaces. As expected, some of the mutants isolated were non-motile (Table 2, column 2). However, most of the strains were as motile as the wild type, yet had severe defects in the initiation of biofilm formation.

Many bacteria, including *P. fluorescens*, synthesize siderophores, phenazines, and other pigments. One of the *sad* mutants (*sad-21*) did not produce this strain's characteristic yellow-green pigment.

Table 2. Phenotypes and molecular analysis of selected *sad* mutants.

Allele	Biofilm Formation on PVC[a]	Motility	Pigment Production	Biofilm Formation: Rescue by Fe, Citrate & Glutamate[b]	Locus[c]
sad$^+$ (wild type)	+	+	+	+	na
sad-10	-	+	+	+	no match
sad-11	-	+	+	+	*clpP*
sad-13	-	-	+	+	*fliP*
sad-14	-	-	+	+	*flaE*
sad-16	-	-	+	+	nd
sad-18	-	+	+	-	no match
sad-19	-	+	+	-[d]	no match
sad-20	-	-	+	+	nd
sad-21	-	+	-	+	nd
sad-22	-	-	+	+	nd

[a]The medium used was M63 minimal medium supplemented with glucose and CAA.

[b]Rescue of the biofilm formation defect was assessed by growing the mutants on M63 minimal medium supplemented with citrate or glutamate at 0.4 %, or M63 minimal medium supplemented with glucose, CAA and 3 mM $FeSO_4$.

[c]The locus was determined by sequencing the DNA flanking the insertion element as described in the Experimental Procedures. If the flanking sequence was homologous to a known locus it is listed. "No match" indicates no significant similarity to any sequence on the database using the BLASTX program (Altschul, S.F., et al., 1990. *J. Mol. Biol.* 215: 403-410). Abbreviations: na, not applicable; nd, not determined.

[d]The biofilm formation defect of *sad-19* is rescued by the addition of citrate, but not by iron or glutamate.

Molecular characterization of *sad* mutants.

The DNA sequence flanking the insertion elements was determined for 24 of the 37 mutants (22 motile and 2 non-motile) in order to identify the gene(s) disrupted in each of the mutants. Typically, 200-400 bp of DNA sequence flanking the transposon insertions were obtained using the arbitrary PCR method. DNA flanking sequences were compared to sequences in Genbank using the BLASTX program (Altschul, S.F. et al., 1990, *J. Mol. Biol.* 215: 403-410). BLASTX translates the DNA sequence in all six reading frames and compares the translated sequences to sequences in Genbank. The results from analyses of selected mutants are presented in Table 2, column 6. Gene identifier sequences of selected *sad* mutants are shown in Fig. 9.

The mutants fall into three broad groups. The first group is comprised of motile strains having their mutation in a locus of known or proposed function. The strain carrying allele *sad-11* (*clpP*) comprises this class. The second group is comprised of non-motile strains, two of which were shown to have mutations in genes required for flagellar synthesis. The third group is comprised of motile strains, but unlike the first group, the DNA sequence flanking the transposon has no obvious similarity to any genes of known function in Genbank, as judged by the BLASTX program. This group of mutants included those having sequences that matched nothing in Genbank and those having sequences that matched genes of unknown function. Transposon insertions from two representative strains of this third group (*sad-18* and *sad-19*) were cloned and over 500 bp of DNA sequence flanking the transposon were determined. Again, no significant matches to genes of known function were found. In fact, only 3 of the 24 mutants analyzed had mutations in genes of known function. Two of these were non-motile mutants (*sad-13* and *sad-14*), in which matches to genes known to be required for synthesis of functional flagella were identified. Taken together,

these results suggest that this genetic screen has allowed us to identify heretofore unknown genes.

Motility is conditionally required for biofilm formation.

Strains carrying alleles *sad-13* and *sad-14* (mutants originally isolated on minimal glucose/CAA medium) have transposon insertions in structural genes required for flagellar synthesis. It appears that we have identified the *P. fluorescens* homolog of *fliP*. The identification was made based on the degree of similarity of the predicted polypeptide encoded by the DNA sequence flanking the insertion in the strain carrying allele *sad-13* to the *P. aeruginosa* PAK *fliP* gene (56% identity and 66% similarity over 77 aa). FliP is thought to participate in flagellar synthesis (Malakooti, J., et al., 1994, *J Bacteriol.* 176: 189-197) and is within an operon containing other flagellar biosynthetic genes, including *fliO*, which is required for non-pili mediated attachment to eukaryotic cells. Because *fliP* is probably part of a gene cluster required for flagellar synthesis, it is not presently possible to conclude whether *fliP* and/or a downstream gene is responsible for the biofilm formation defect. The strain carrying allele *sad-14* contains a insertion in what appears to be the *P. fluorescens* homolog of the *flaE* gene of *Vibrio paramaemolyticus* (McCarter, L.L.,1995, *J. Bacteriol.* 177: 1595-1609) and the *flgK* gene of *Salmonella typhimurium* (Homma, M. et al.,1990, *J. Mol. Biol.* 213: 819-832). The predicted polypeptide (~70 aa) encoded by the sequence flanking the insertion in *sad-14* is ~40% identical and ~60% similar to the *flaE* and *flgK* genes. These genes are thought to encode a structural component of the flagellum. The isolation of multiple non-motile mutants that are also defective for biofilm formation on an abiotic surface shows that there is an overlap between factors required for biofilm formation on biotic and abiotic surfaces, and further validates our approach for isolating mutants defective in this

process. As shown below, however, flagella-mediated motility only appears to be required under certain growth conditions.

The Clp protease participates in biofilm formation.

The DNA sequence flanking the insertion in the strain carrying *sad-11*, which is motile and is defective in forming biofilms on both hydrophilic and hydrophobic surfaces, encodes a polypeptide with high similarity (~80% identity and ~95% similarity over a 54 aa stretch) to the ClpP protein of *E. coli*, which is a subunit of the cytoplasmic Clp protease (Gottesman, S. and Maurizi, M.R., 1992, *Microbiol. Rev.* 56: 592-621). Based on this level of similarity, we propose that we have identified the ClpP protein homolog of *P. fluorescens*. The location of the transposon insertion in *clpP* is just downstream of the putative start of translation.

We performed complementation analysis to confirm that the mutation in *clpP* was causing the biofilm formation defect. The *clpP* gene of *E. coli* was amplified from chromosomal DNA of ZK126 (W3110 *clpP*$^+$) by PCR and cloned into a vector (pSMC28) that is stably maintained in *Pseudomonas* spp. The resulting plasmid pSMC26 (*clpP*$^+$), and the vector control (pSMC28), were introduced into wild-type *P. fluorescens* and the *sad-11* (*clpP*) mutant. These plasmid-carrying strains were then tested for biofilm formation. These data are summarized in Fig. 5. The first two columns of Fig. 5 show the biofilm formation phenotype of the wild-type and *clpP* strains (not carrying any plasmids). Complementation analysis (columns 3-6) revealed that the biofilm formation of the *clpP* mutant is completely rescued by providing a plasmid-borne copy of *clpP*$^+$ derived from *E. coli* (column 5). The vector control has no effect on biofilm formation of the wild-type or *clpP* strain (columns 3 and 4).

Providing *clpP* in multiple copies appears to have no adverse effects on biofilm formation by the wild-type strain.

We also directly assessed the ability of the *clpP* mutant carrying pSMC26 (*clpP*$^+$) or the vector control (pSMC28) to attach to PVC, using phase contrast microscopy (Fig. 6; 600X magnification; assays performed in minimal glucose/CAA medium). The left panel of Fig. 6 shows multiple cells adhered to the PVC plastic when the *clpP* mutant is carrying pSMC26 (*clpP*$^+$). This phenotype is similar to what is seen with the wild-type strain. When the *clpP* mutant carries just the vector control (Fig. 6, right panel) very few cells are found attached to the PVC plastic. These data are consistent with the indirect assessment of biofilm formation by CV-staining that are shown in Fig. 5, and demonstrate that the ClpP protein participates in biofilm formation.

Multiple signaling pathways participate in biofilm formation.

As discussed above, various nutritional conditions impact biofilm formation by *P. fluorescens*. Based on these observations, biofilm formation by the *sad* mutants (originally isolated on minimal medium supplemented with glucose and CAA) was assessed in a variety of media. The biofilm formation defect of approximately half of the *sad* mutants was rescued by growth on minimal medium supplemented with citrate or glutamate as the sole source of carbon and energy, or minimal glucose/CAA medium supplemented with 3 μM $FeSO_4$.

Fig. 7 shows rescue of the biofilm formation defect of *sad* mutants. The extent of biofilm formation after 10 hrs of growth is expressed as the absorbance at 600 nm. Shown are the values for the wild type and selected *sad* mutants. The biofilm formation phenotype of the *sad* mutants was assessed with

cells grown on minimal medium supplemented with: (i) glucose/CAA, glucose/CAA plus iron (3 mM), (ii) citrate (0.4%), or (iii) glutamate (0.4%).

The *sad* mutants could be divided into three classes based on their ability to be rescued by citrate, glutamate or iron-supplemented glucose/CAA medium (Fig. 7 and Table 2). One class (containing 12 mutants) represented by the strain carrying allele *sad-18*, showed a strong biofilm formation defect under all nutritional conditions tested. The second class, represented by the single strain carrying the *sad-19* allele, was rescued by growth on citrate, but not on glutamate or glucose/CAA + iron. The remainder of the *sad* mutants (10 mutants) were rescued for their biofilm formation defect when grown on minimal medium supplemented with citrate, glutamate, or glucose/CAA + iron.

Among the *sad* mutants rescued by growth on citrate, glutamate, or glucose/CAA + iron medium were the non-motile strains shown to carry mutations in the genes required for flagellar synthesis (see Table 2). It is important to note that growth on citrate, glutamate or iron-supplemented glucose medium, while restoring the cells' ability to form biofilms, does not restore motility as assayed on 0.3% motility agar plates. Furthermore, although 0.29% malate, mannitol, xylose, and glycerol promote biofilm formation 0.2%, these carbon sources did not rescue the biofilm formation defect of any of the *sad* mutants. Therefore, rescue of the biofilm formation defect was specific for particular growth conditions.

The growth of mutants in minimal glucose/CAA medium supplemented with $CaCl_2$, $MgSO_4$, and $MnSO_4$ (all provided at 3 μM) did not restore their ability to form biofilms, indicating that the ability to rescue the biofilm formation defect of the *sad* mutants is specific to iron. Taken together, these data show that multiple, convergent genetic pathways are involved in the

early events of biofilm formation and these pathways can be induced by various, specific environmental signals.

Example III: Identification of mutations that affect biofilm formation in *Escherichia coli*

E. coli Forms Biofilms in a Nutrient-dependent Fashion.

We tested the ability of the well characterized, gram-negative bacterium, *E. coli*, to initiate biofilm formation on abiotic surfaces. To assay for such attachment, we used a modified version of a previously described protocol (Fletcher, M.,1977, *Can. J. Microbiol.*, 23, 1-6). Cells were first grown for either 24 or 48 hours at room temperature without shaking in microtiter dishes or glass tubes. In order to remove any unattached cells, the microtiter dishes (or glass tubes) were rinsed thoroughly with water and subsequently stained with 1.0% crystal violet (CV) for approximately 20 minutes. This staining procedure allowed us to visualize cells that had attached to an abiotic surface because attached cells stain purple with CV whereas abiotic surfaces are not stained by CV. We found that a number of motile laboratory strains of *E. coli* were able to attach to multiple abiotic surfaces when grown in Luria Bertani broth (LB). Specifically, *E. coli* W3110 formed biofilms on all surfaces tested, including polyvinyl chloride (PVC), polypropylene, polycarbonate, polystyrene, and borosilicate glass.

Importantly, the ability to form such biofilms was strongly influenced by the nutritional environment. Figure 10 shows the nutritional effects on biofilm formation. Wild-type cells were grown in PVC microtiter dishes in LB at room temperature without shaking for 24 hours, then subcultured (1:100) into PVC microtiter dishes containing the indicated media. These cultures were

grown for 48 hours at room temperature without shaking, then rinsed and stained with crystal violet. Biofilm formation could be visualized with CV after as little as two hours of growth in LB. Similarly, biofilm formation was supported by various minimal media containing casamino acids (CAA) (Fig.10). In contrast, minimal media without CAA (with either glucose or glycerol as a carbon and energy source) did not support biofilm formation that was visible after staining with CV (Fig.10).

Screen for *E. coli* Mutants Defective in Biofilm Formation.

To identify genes required for biofilm formation, we screened for mutants defective in forming biofilms in LB on PVC plastic. Strain W3110 was subjected to insertion mutagenesis (Kleckner, et al., *supra*) with a mini Tn10cam, and insertion mutants were selected on LB agar containing 30 μg/mL chloramphenicol.

Chloramphenicol resistant colonies were picked and grown at room temperature in 96-well PVC microtiter dishes containing glucose minimal medium with 30 μg/ml chloramphenicol. After 48 hours, the cells were subcultured into corresponding wells in a 96-well PVC microtiter dish containing LB with 30 μg/mL chloramphenicol. The cultures were grown at room temperature for another 48 hours and then rinsed thoroughly with water to remove any planktonic cells. The wells were stained with CV, rinsed, and potential biofilm-defective mutants were identified based on decreased staining compared to a wild-type control. Each potential biofilm-defective mutant was isolated from its original microtiter well, streaked for single colonies on LB agar, and re-tested for its ability to form a biofilm. Each of the insertion mutations that appeared to confer a defect in biofilm formation was transferred into a fresh W3110 background via P1vir-transduction and re-tested. Of 10,000 such

insertion mutations analyzed, 177 were found to confer a decrease in biofilm formation.

Initial Classification and Mutant Identification.

It is possible that a mutant strain isolated in the above screen could exhibit decreased biofilm formation because it harbors a mutation that either: (1) confers a non-specific growth defect that indirectly affects biofilm development, or (2) interferes in the formation of biofilms without interfering with the growth rate. To distinguish between these possibilities, mutant strains were grown in LB and their growth rates were compared to the wild type. Only strains exhibiting growth rates indistinguishable from the wild type are discussed below.

The mutant strains displayed a wide array of phenotypes with respect to the severity in their decreased ability to form biofilms. The macroscopic phenotypes ranged from wells that displayed subtle decreases in CV staining to wells that appeared completely clear after CV treatment. As an early step in characterization of the mutants, each was analyzed for its ability to swarm on LB motility agar (0.3% agar). Approximately one-half of the mutants (87/177) displayed a decreased ability to swarm, whereas the remaining mutants formed swarms that were indistinguishable from the wild type. The majority of the Swarm$^-$ mutants were severely defective in their ability to form biofilms (i.e. clear wells after staining with CV). Such swarm assays do not always allow one to distinguish between defects in flagellar biosynthesis, motility, and/or chemotaxis. Thus, the following central question arose: Which of these three aspects of bacterial flagella/movement is critical to biofilm formation?

Among the remaining Swarm$^+$ mutants, 23 displayed macroscopic phenotypes comparable to those observed with Swarm$^-$ mutants (i.e. clear wells after staining with CV; see examples of mutants that display the clear well

phenotype in Fig. 11), whereas the others displayed less severe phenotypes. In this initial study, we focused on the 23 Swarm$^+$ mutants with the strongest phenotypes. The 23 mutants referred to above were found to be tightly linked to each other, as indicated by P1vir-transduction using a nearby Tn10. The precise locations of nine of the 23 insertion mutations within this linkage group were identified utilizing arbitrarily primed PCR followed by DNA sequence analysis. All nine insertions were located in genes encoding for the regulation or synthesis of type I pili. Specifically, independently isolated insertions were found in *fimB* (two alleles), *fimA*, *fimC*, *fimD* (three alleles), and *fimH*. Thus, a second question arose: What is the role of type I pili in *E. coli* biofilm formation?

Motility, not Chemotaxis, is Critical for Biofilm Formation.

We reasoned that there are three mechanisms through which flagella might be required for biofilm formation. First, it is possible that flagella could be directly required for attachment to abiotic surfaces, thus facilitating the initiation of biofilm formation (e.g. as with tethered cells). Alternatively, motility could be necessary to enable a bacterium to reach the surface (e.g. to move through surface repulsion present at the medium-surface interface). Also, motility might be required for the bacteria within a developing biofilm to move along the surface, thereby facilitating growth and spread of the biofilm. Finally, it is possible that chemotaxis is required for the bacteria to swim towards nutrients associated with a surface.

Since flagellar synthesis, motility, and chemotaxis have been extensively studied in *E. coli* (Macnab, R.M.,1996, In Neidhardt, F.C., et al. (ed.), *Escherichia coli and Salmonella typhimurium: Cellular and molecular biology* ASM Press, Washington, DC, Vol. 2, pp. 123-145; Stock, J.B. and Surette, M.G., 1996, In Neidhardt, F.C., et al. (ed.), *Escherichia coli and*

Salmonella typhimurium: Cellular and molecular biology ASM Press, Washington, DC, Vol. 2, pp. 1103-1129), well defined mutations that inhibit each of these three aspects of flagellar function are available. Accordingly, we obtained the following mutations: 1. *fliC::kan* (strains harboring this allele are unable to synthesize flagellin) and *flhD::kan* (a master regulator of flagellar synthesis whose absence confers an inability to synthesize flagella), 2. *DmotA*, *DmotB* and *DmotAB* (lesions that do not inhibit flagellar biosynthesis, but render cells non-motile or paralyzed), 3. *DcheA-Z::kan* (strains harboring this lesion are motile, but non-chemotactic).

Each of these alleles was moved into W3110 via P1vir-transduction, and the resulting strains were analyzed for their ability to form biofilms. Construction of these strains provided us with the tools required to distinguish between the possible roles of flagella/motility/chemotaxis that were detailed above. Fig. 11 shows biofilm formation of wild-type and mutant strains. Cells with the indicated genotypes were grown in PVC microtiter dishes in LB at room temperature without shaking for 24 hours, then subculture (1:50) into LB. These cultures were grown for 24 hours at room temperature without shaking, then rinsed and stained with crystal violet. This assay revealed that motile cells that are non-chemotactic (*DcheA-Z::kan*) appear to form biofilms indistinguishable from their wild-type counterpart. In contrast, cells either lacking flagella (*fliC::kan* , *flhD::kan*) or possessing paralyzed flagella (DmotA, DmotB, or D motAB) were severely defective in biofilm formation (Fig. 11).

Fig. 12 shows quantification of biofilm formation. Cells with the indicated genotypes were grown for 24 hours in PVC microtiter dishes containing LB, then subcultured (1:50) into PVC microtiter dishes with LB. At the times indicated, the microtiter dishes were rinsed, stained with CV, and the amount of CV staining was quantified. When biofilm formation was quantified

over time, it became very clear that, under these conditions, chemotaxis is completely dispensable for normal biofilm formation (Fig. 12). In contrast, cells either lacking complete flagella (*fliC::kan*) or possessing paralyzed flagella (*DmotA*, *DmotB*, or *DmotAB*) are severely hindered in the initial stages of biofilm formation (Fig.12).

More detailed analysis of the defects conferred by these alleles was obtained through microscopic analysis of cells attached (or the absence of such attached cells) to PVC following growth in LB Fig 13 (A-D). Cells with the indicated genotypes were grown in PVC microtiter dishes in LB at room temperature without shaking for 24 hours, then subculture (1:50) into microtiter dishes containing LB and a tab of PVC plastic. These cultures were grown for 24 hours at room temperature without shaking. The PVC tabs were then removed, rinsed, and the remaining cells were visualized via phase contrast microscopy (400X magnification). Panel A shows the wild-type strain W3110; Panel B shows the mutant strain W3110 DcheA-Z::kan (which is non-chemotactic); Panel C shows the mutant strain W3110 FimH1::cam (which lacks pili); and Panel D shows the mutant strain W3110 flhD::kan (which lacks flagella). As illustrated in Fig. 13B, motile cells that are non-chemotactic are able to form biofilms that are indistinguishable at the cellular level from the biofilms formed by wild-type cells. In contrast, non-flagellated or paralyzed cells attach poorly to PVC. Moreover, the few cells that do attach are often located in small, dense clusters of cells (Fig. 13D).

Type I Pili are Critical for Initial Attachment to Abiotic Surfaces.

As discussed above, the macroscopic analysis of biofilm formation of *fim* mutants was analogous to that observed with the motility defective mutants (i.e. clear wells after staining with CV) (Fig. 11). However, microscopic analysis

of these mutants revealed distinct phenotypes. Specifically, *fim* mutants are even more dramatically defective in initial attachment than are the paralyzed and non-flagellated cells. As illustrated in Figure 13C, in most microscopic fields no attached cells were observed, and only infrequently were a few attached cells observed. This result indicates that type I pili are critical for initial interaction with abiotic surfaces such as PVC.

α-Methyl-D-Mannoside Inhibits Attachment to Abiotic Surfaces.

One of the insertions in the *fim* gene cluster is located in the final gene if the operon, *fimH*. Lesions in *fimH* have been reported to affect the length of the tip (fibrilla) of type I pili (Ottemann, K.M. and Miller, J.F., 1997, *Mol. Microbiol.*, 24, 1109-1117). In addition, *FimH* functions as a mannose-specific adhesion, allowing *E. coli* to interact specifically with mannose residues on eukaryotic cells, thus facilitating infections such as cystitis (Hanson, M.S. and Brinton, C.C.,1988, *Nature*, 332, 265-268.; Low, D et al, 1996, In Neidhardt, F.C., et al. (ed.), *Escherichia Coli and Salmonella Typhimurium: Cellular and Molecular Biology* ASM Press, Washington, D.C., Vol. 1, pp. 146-157.; Maurer, L. and Orndorff, P.,1987, *J. Bacteriol,* 169, 640-645; Old, D.C., 1972. *J. of Gen. Microbiol.*, 71, 149-157). Consequently, it is possible that the altered structure of the fibrilla of type I pili in *fimH* mutants could interfere with normal attachment to abiotic surfaces. Alternatively, the mannose-specific adhesin may play a more direct role in attachment.

To further address the role of *FimH* in biofilm formation, we tested whether the presence of a non-metabolizable mannose analog, α-methyl-D-mannoside, affected the ability of the wild-type strain, W3110, to form biofilms on PVC. Fig. 14 shows the effects of α-methyl-D-mannoside on biofilm formation. Cells were grown for 24 hours without shaking at room

temperature, and then subcultured (1:50) into PVC microtiter dishes with LB plus 0, 5, 15, 25, 50, or 100 mM α-methyl-D-mannoside. After nine hours at room temperature without shaking, the microtiter dishes were rinsed, stained with CV, and the amount of CV staining was quantified. As illustrated in Fig.14, α-methyl-D-mannoside inhibits biofilm formation in a concentration-dependent fashion. Importantly, α-methyl-D-mannoside does not inhibit growth rates. As a specificity control, we have shown that although mannose also has a similar effect as α-methyl-D-mannoside, glucose does not inhibit biofilm formation, and neither mannose nor glucose inhibits growth. It is also important to note that α-methyl-D-mannose inhibits biofilm development on all other abiotic surfaces tested, including polycarbonate, polystyrene, and borosilicate glass. It is reasonable to assume that these various surfaces do not resemble mannose.

Fig. 15 shows a model for initiation of *E. coli* biofilm formation. Motility may be required to overcome surface repulsion, thereby allowing initial surface contact. Type I pili are needed to establish stable attachment, perhaps through interactions between the type I adhesion, *FimH*, and the abiotic surface. Finally, motility may also enable attached, growing cells to migrate along the abiotic surface, thereby facilitating biofilm expansion.

Example IV: Identification of mutations that affect biofilm formation in *Pseudomonas aeruginosa*

Isolation of mutants defective in biofilm formation.

We generated a collection of ~2400 random transposon mutants of *P. aeruginosa* PA14 using the transposon Tn5-B30(Tcr) (Simon, R. et al., 1989, *Gene*, 80, 160-169). This collection of *P. aeruginosa* mutants was screened in microtiter dishes made of polyvinylchloride (PVC) to test for their ability to form

a biofilm on an abiotic surface. The cells were allowed to grow in the wells of the microtiter dishes in a minimal M63 medium supplemented with glucose and casamino acids (CAA) to assess their ability to form a biofilm, as described above in the previous Examples. The biofilm was detected by staining with crystal violet (CV), a purple dye which stains the bacterial cells, but does not stain the PVC plastic. After addition of CV and incubation at room temperature for ~10 min, excess CV and unattached cells were removed by vigorous and repeated washing of the microtiter plates with water. An example of the phenotype of the wild-type strain is shown in Fig. 16. The biofilm is observed as a ring of CV-stained cells which forms at the interface between air and medium. Under the growth conditions used in this experiment, the only electron acceptor available is oxygen. Therefore, the biofilm forms only where oxygen levels are highest, that is, at the interface between air and medium. Of the ~2400 mutants screened, 15 mutants (0.5%) unable to form such a biofilm were isolated. These mutants were designated *s*urface *a*ttachment *d*efective or *sad*. The biofilm formation phenotypes of representative *sad* mutants pilY1 (genbank (gb) accession no. L76605), *pilB* (gb-M32066), and *flgK* (gb-X51738) are also shown in Fig. 16.

Any strains exhibiting poor growth under these screening conditions might give the same phenotype as mutants unable to initiate formation of a biofilm. Therefore, all of the putative *sad* mutants were grown in liquid minimal M63 medium supplemented with glucose and CAA (the same medium used to screen for mutants). Of the 15 putative *sad* mutants tested, 13 grew as well as the wild-type strain, but were unable to form a biofilm. The other two putative *sad* mutants had severe growth defects relative to the wild type and were not analyzed further.

We performed Southern blot analysis of the 13 *sad* mutants that did not form a biofilm to determine the number of transposon insertions in each strain. A PCR-generated DNA fragment from the IS50 of Tn5 was used to probe EcoRI-digested chromosomal DNA (there are no EcoRI sites in Tn5-B30). This analysis revealed a single hybridizing band for each strain, consistent with each *sad* mutant having only a single transposon insertion. The further analyses of two classes of mutants (totaling 8 of 13) isolated in this screen is presented below.

We tested the *P. aeruginosa sad* mutants for their ability to form a biofilm on abiotic surfaces other than PVC, including polystyrene, polycarbonate and polypropylene. The wild-type strain can form a biofilm on all of these surfaces. In contrast, all of the *sad* mutants originally isolated on PVC were also defective for biofilm formation on these other surfaces.

Non-motile mutants are defective in biofilm formation.

In addition to the phenotypic analyses described above, all *sad* mutants were assessed for their motility phenotype on 0.3% agar (minimal M63 medium supplemented with glucose and CAA). Fig. 17 shows an example of a motility assay. The flagella-mediated motility of the wild-type strain, representative pili-defective mutants (*pilB* and *pilC*), and non-motile mutants (*flgK*, *sad-39*, and *sad-42*) was assessed on minimal M63 glucose/CAA medium with 0.3% agar after ~24 hrs of growth at 25°C. Migration of the cells from the point of inoculation (observed as a turbid zone) indicates that the strain is proficient for flagellar-mediated motility.

Of the 13 mutants tested, three strains (*sad-36*, *sad-39*, and *sad-42*) were found to be non-motile (Fig. 17). In a typical experiment after 24 hrs of growth at room temperature, the wild type and two representative mutants

defective in pili biogenesis (*pilB* and *pilC*) clearly migrated from the point of inoculation while the *sad-36*, *sad-39*, and *sad-42* strains did not.

One of these mutants, *sad-36*, was chosen for further analysis. The *sad-36::Tn5(Tcr)* insertion was mobilized into a wild-type genetic background by phage SN-T-mediated transduction as reported (Jensen, E.C., et al., 1998, *Appl. Environ. Microbiol.*, 64, 575-580). 18 of 18 *Tcr* transductants (indicating inheritance of the Tn5 element) were non-motile and unable to make a biofilm, demonstrating that the single insertion in this strain was responsible for the observed phenotypes. The DNA sequence flanking the Tn5 insertion in *sad-36* was determined using arbitrary PCR and compared to the Genbank database using BLASTX (Altschul, S.F., et al., 1990, *J. Mol. Biol.* 215: 403-410). BLASTX translates DNA sequence in all six reading frames and compares these predicted protein sequences to Genbank. The determined DNA sequence flanking the Tn5 element (~375 nt), when translated, revealed a partial ORF with ~40% identity and ~65% similarity to HAP1 (*flgK*), the flagellar-associated hook protein 1 of *Salmonella typhimurium* and *Escherichia coli*. Mutations in the *flgK* locus in these organisms results in the synthesis of an incomplete flagellum, which renders the strains non-motile (Homma, M., et al., 1990, *J. Mol. Biol.*, 213, 819-832). The localization of the Tn5 insert of the strain carrying the *sad-36* allele to a gene required for flagellar function is consistent with the non-motile phenotype of this strain.

Type IV pili are required for biofilm formation.

We analyzed the DNA sequence flanking the transposon inserts of the other *sad* mutants. Comparison of the translated DNA sequences flanking the Tn5 insertions in *sad* mutants to the Genbank database revealed that five strains carried mutations in genes required for the synthesis of type IV pili.

Type IV pili are important for the adherence to and colonization of eukaryotic cell surfaces and are thought to play a role in pathogenesis. Four of the five mutants defective in type IV pili biogenesis identified in the screen had mutations in the *pilBCD* operon, which is thought to code for accessory factors required for pili assembly and function. The strains carrying alleles *sad-31*, *sad-33*, and *sad-34* have mutations in the *pilB* gene. The DNA sequence flanking the transposon insertions in *sad-33* and *sad-34* was identical, indicating that these two strains were probably siblings. The mutations carried in *sad-31* and *sad-33/sad-34* map to two different locations within *pilB*.

The strain carrying allele *sad-29* has a mutation in the *pilC* gene. Because the *pilBCD* locus may form an operon, it is possible that polarity onto *pilD* is actually causing the phenotype. However, it has been shown in *P. aeruginosa* PAO1 that mutations in any of these loci result in the loss of the synthesis of pili as indicated by resistance to the pilus-specific bacteriophage PO4 and visual inspection by electron microscopy. (Nunn, D., et al., 1990, *J. Bacteriol.*, 172, 2911-2919).

The fifth mutant, *sad-25*, maps to yet a third locus, a homolog of the *pilY1* gene of *P. aeruginosa* PAO1. In *P. aeruginosa*, the *pilY1* gene is in a cluster of genes (including *pilV*, *pilW*, *pilX*, *pilY2*, and *pilE*) that are required for type IV pili biogenesis. Consistent with the mapping of these mutations to genes required for type IV pili biogenesis was their resistance to lysis by phage F116 (Pemberton, J.M., 1973, *Virology*, 55, 558-560), which utilizes type IV pili as its receptor.

It has been shown that type IV pili are required for a form of surface-associated movement known as twitching motility. Twitching motility is thought to be a consequence of the extension and retraction of type IV pili, which

propels the bacteria across a surface by an undescribed mechanism (Bradley, D.E., 1980, *Can. J. Microbiol.*, 26, 146-154; Darzins, A., 1994, *Mol. Microbiol.*, 11, 137-153.; Whitchurch, C.B., 1990. *Gene,* 101, 33-44). We assessed the twitching motility phenotype of the mutants carrying alleles *sad-25* (*pilY1*), *sad-29* (*pilC*), *sad-31* (*pilB*), and *sad-33* (*pilB*). The wild-type, a representative flagellar mutant (*flgK*), and four type IV pili mutants are shown in Fig. 18.

To assess twitching motility, cells were stabbed into an LB agar plate (1.5% agar) with a toothpick, incubated overnight at 37°C, then for 1-2 days at room temperature (~25°C). Twitch$^+$ strains form a colony on the agar surface and form a hazy zone of cell growth within the agar substrate. Twitch$^-$ strains still form a colony on the agar, but lack the zone of growth within the agar. Also, the colonies of Twitch$^+$ strains are flat, spreading, and irregularly shaped, while the colonies formed by strains defective in the synthesis of type IV pili are rounded and somewhat dome-shaped.

In addition to forming a colony on the surface of the agar plate (1.5% agar), Twitch$^+$ strains of *P. aeruginosa* PA14 form a haze of growth that surrounds the point of inoculation. This assay differs from the test for flagella-mediated motility, which is performed by inoculating cells onto 0.3% agar plates (see Fig. 17). Furthermore, strains capable of twitching motility have a spreading colony morphology while strains defective in twitching motility produce rounded colonies. This difference in colony shape can also be observed in Fig. 18.

Twitching motility can also be assessed by phase-contrast microscopy. At the microscopic level, the edge of the colonies of strains proficient in twitching motility are highly irregular. This is thought to be a consequence of the surface movement associated with type IV pili. Mutants lacking functional

type IV pili have smooth-edged colonies. To further confirm that our strains did not have functional type IV pili, we observed the edges of wild-type and pili-deficient mutants by phase contrast microscopy. As shown in Fig. 19 (micrographs are at 400X magnification), the wild-type strain has the expected irregular colony edge and the representative pili-deficient strain (*sad-31/pilB*) has the expected smooth colony edge phenotype. All the pili-defective mutants behaved in a fashion identical to *sad-31*. Transmission electron microscopic analysis of the pili mutants confirmed the lack of these structures on the surface of the mutant cells.

Mutants defective in flagellar-mediated motility and type IV pili biogenesis define two steps in a developmental pathway. We utilized the *sad* mutants isolated in this study as tools to initiate the dissection of the early steps in biofilm formation. In order to follow the initiation of biofilm formation by the wild-type and *sad* mutants, we directly visualized the formation of the biofilm on PVC using phase contrast microscopy. A small tab of PVC plastic (~3mm x ~6mm) was incubated in the well of a microtiter dish that had been inoculated with 10^6 CFU/mL of the appropriate strain in minimal M63 medium supplemented with glucose and CAA. After incubation for various times at 37°C, the plastic tab was removed from the microtiter dish with ethanol-sterilized forceps, rinsed with 1 mL of sterile minimal M63 medium, placed on a slide, and examined by phase-contrast microscopy (400X magnification).

Fig. 20 shows a time course of the development of a biofilm on PVC by the wild-type strain over 7.5 hrs at 37°C as observed by phase-contrast microscopy. As early as 30 minutes after inoculation, the wild type formed a dispersed monolayer of bacterial cells attached to the surface of the PVC plastic. A progressively more dense monolayer of cells formed on the surface over the next 3-4 hours. By 5 hours, and continuing until at least 7.5 hours, this

monolayer almost completely covered the PVC surface and became punctuated by micro-colonies (indicated by arrows) which were distributed across the surface of the PVC plastic and were comprised of multiple layers of cells. Typically, the wild-type micro-colonies were ~3-5 layers of cells thick.

We directly visualized the ability of the type IV pili-deficient and non-motile strains to form a biofilm on PVC using phase-contrast microscopy and compared their phenotypes to the wild-type strain. Fig. 21 shows phase-contrast photomicrographs of the wild-type strain, a representative pili-defective mutant (*flgK*), and a representative non-motile mutant (*pilB*) after incubation for 3 hours at 37°C in the presence of PVC plastic. Micrographs were taken at 400X magnification; approximately 50 fields were searched for each strain tested, and representative fields are shown. For the representative non-motile strain (carrying a mutation in *flgK*), few to no cells were observed attached the PVC plastic even after 8 hrs of incubation in the presence of the PVC surface (Fig. 21). All other non-motile strains analyzed had a phenotype identical to the *flgK* mutant.

We also directly visualized the biofilm formation phenotype of a representative mutant defective in pili biogenesis (*pilB*). At the early time points (< 3 hrs), there was little difference in the biofilm formation phenotype of the wild type and the type IV pili mutants; both the wild-type and the pili-defective strain form a dispersed monolayer of cells on the surface of the PVC plastic. By 8 hours, in contrast to the aggregates of cells formed by the wild-type strain, the pili-defective mutants did not develop these characteristic micro-colonies (Fig. 21). Furthermore, the wild-type strain almost completely covered the PVC surface with a dense, tightly-packed layer of cells (Fig. 21). The phenotype of the type IV pili mutants at this 8 hour time point was unchanged from that

observed at 3 hrs, that is, a dispersed monolayer of cells. The other mutants defective in pili biogenesis (*pilC* and *pilY1*) had similar phenotypes.

A role for twitching motility in biofilm formation.

To better define the events that lead to micro-colony formation by the wild type and to determine if surface-based twitching motility plays a role in biofilm formation, we employed phase-contrast time-lapse microscopy to follow a developing biofilm. Utilizing time-lapse microscopy, we watched individual micro-colonies formed by the wild-type strain over a period of 56 minutes (with images acquired at 15 second intervals). Shown in Figs. 22A-22I is a montage of 9 phase-contrast micrographs taken during biofilm formation by the wild-type strain every 7 minutes between 360 and 416 minutes post-inoculation. Arrows indicate micro-colonies that form and/or disperse over the course of the experiment. The black circles indicate the identical spot on the field in panels H and I. Several micro-colonies were followed through the course of this experiment to illustrate the movement of cells across the PVC plastic surface.

In Figs. 22A-22I, the white arrow indicates the position of a micro-colony which is first clearly visible in Fig. 22B, becomes larger (Fig. 22C), but has dispersed by Fig. 22D. This micro-colony does not reform during the course of this experiment (Figs. 22D through 22I). A series of time-lapse micrographs taken at 15 second intervals between 374 minutes (Fig. 22C) and 381 minutes (Fig. 22D) show that this micro-colony disperses because the cells comprising the colony move apart, while still remaining associated with the plastic surface.

The black arrow points to a large micro-colony evident in Fig. 22A. This large micro-colony becomes progressively smaller (Figs. 22B through 22F) and eventually splits into two small, adjacent micro-colonies (Fig. 22G). In Fig.

22H, these two adjacent micro-colonies form a larger single colony which has grown slightly in size when visualized 7 minutes later (Fig. 22I).

The formation of micro-colonies in this system is due in large part to the aggregation of cells found dispersed in the monolayer of cells on the surface and not solely to the growth of the bacterial cells. This point is further illustrated by data presented in Figs. 22H and 22I. The dark circle in Fig. 22I indicates a dense, well-formed micro-colony. However, this colony is not evident 7 minutes previously in Fig. 22H. The elapsed 7 minutes between the micrograph shown in Fig. 22H and the micrograph shown in Fig. 22I represents less than the time needed for a single population doubling under these growth conditions. Furthermore, analysis of the time-lapse film shows that this micro-colony forms by recruiting adjacent cells from the monolayer. The data described above and shown in Figs. 22A-22I demonstrate the dynamic nature of micro-colony formation and dispersal during the course of biofilm development.

As discussed above, type IV pili are required for surface based twitching motility and mutants defective in type IV pili biogenesis do not make the micro-colonies characteristic of the wild-type strain. It is important to note that none of the behaviors described above for the wild-type were observed in the representative type IV pili mutant, *pilB*. As shown above in Fig. 21, this strain does not form micro-colonies when observed either after 8 hrs of growth or when monitored by time-lapse microscopy.

Fig. 23 shows a model for the role of flagella and type IV pili in *P. aeruginosa* biofilm formation. Flagella or flagella-mediated motility appear to be important for the formation of a bacterial monolayer of the abiotic surface. Type IV pili appear to play a role in downstream events such as micro-colony formation.

Other Embodiments

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth, and follows in the scope of the appended claims.

What is claimed is: